# OBITUARY NOTICE

## PERCIVAL WALTER CLUTTERBUCK

1897–1938

By the death of Percival Walter Clutterbuck, the ranks of the small band of British biochemists trained in their student days by the late Prof. J. B. Cohen at Leeds, and first broken by the death just over two years ago of Harold Dudley, have suffered a further serious loss.

Clutterbuck was born at Armley near Leeds, and from the local elementary school he passed to the Leeds Central High School, and thence in 1914 to the University of Leeds, where he continued his studies until 1916. In February of that year he volunteered for service in the Royal Engineers, Gas Warfare Section. After a short service in France he was transferred, after being gassed at the Front, to the Gas and Trench Warfare Research Station, Porton, Wilts. Here, in spite of his short university training, he gave such good service during the rest of the War that, at the end of the War, he was awarded the O.B.E. Soon after the close of the War he returned to Leeds, and graduated in 1922, B.Sc. with first class honours in chemistry. He remained at Leeds for some time, carrying out research work with the late Prof. J. B. Cohen, and then went to Manchester in 1923 to Prof. H. S. Raper's laboratory, remaining there until 1928. He graduated M.Sc. in 1923, Ph.D. in 1924 and D.Sc. in 1930. In 1924 he was appointed Demonstrator in Physiological Chemistry to Prof. Raper. Awarded a Beit Memorial Fellowship for Medical Research in 1928, he worked for six months at the Lister Institute under Prof. (now Sir Arthur) Harden, and early in 1929 joined the writer's research staff in the Biochemical Department of Messrs Nobel's Explosives Company, Ltd., Ardeer Factory, Stevenston, Ayrshire. In 1931 this Department was closed down, and Clutterbuck, with one or two others, was seconded to the writer's Department of Biochemistry at the London School of Hygiene and Tropical Medicine, where in October 1933 he was appointed Lecturer in Biochemistry, a post which he held at the time of his death.

Early in December 1937 Clutterbuck was stricken with an acute appendicitis, and following an operation, from the after effects of which he was apparently recovering, he died in Northwood and Pinner District Hospital as the New Year of 1938 was dawning.

Clutterbuck's research career had three distinct phases. In the first phase he contributed two papers jointly with J. B. Cohen, the first on the aliphatic sulphonamides (*J. chem. Soc.* (1922), **121**, 120) and the second on the aryl and alkyl sulphonamides (*J. chem. Soc.* (1923), **123**, 2507). In the second phase, in collaboration with H. S. Raper, Clutterbuck published a series of papers on the oxidation, both *in vivo* and *in vitro*, of a series of organic acids (*Biochem. J.* (1925), **19**, 385, 911; (1926), **20**, 59; (1927), **21**, 512; (1928), **22**, 745, 1193).

When Clutterbuck joined the writer's staff in 1929 he immediately threw himself wholeheartedly into the study of the biochemistry of a large series of micro-organisms, and this work he continued with increasing enthusiasm until his death. While at Ardeer he was mainly concerned with the preparation of

carbon balance sheets for a large number of different species of bacteria (*Biochem. J.* (1931), **25**, 1522), and for the last six years of his life he proved an ideal collaborator with the writer on the biochemistry of a number of species of fungi. The results obtained were communicated in a series of papers appearing in this *Journal*, and the writer desires to pay tribute to Clutterbuck's capabilities as a research worker, as a supervisor of young research students and above all as a loyal friend and a faithful colleague.

He married in 1926 Muriel North, daughter of Mr Barker North, late Head of the Chemical Department, Bradford Technical College. His widow and two young daughters survive him. H. R.

# LVI. STUDIES OF THE METABOLISM OF THE STRICT ANAEROBES (GENUS: *CLOSTRIDIUM*)

## I. DEHYDROGENATION REACTIONS BY SUSPENSIONS OF *CL. SPOROGENES*

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(*Received 24 December 1937*)

Our knowledge of the metabolism of the spore-forming anaerobes has until now been rather restricted. This is due in part to the fact that from the biochemical point of view little attention has been given to this genus. This is regrettable, since this group of micro-organisms has the unique property of being able to live and multiply actively under anaerobic conditions with proteins as the only source of energy. A study of the metabolism of these organisms, therefore, may yield valuable information on protein metabolism in general.

Previous investigations consisted mainly in the detection of dissimilation products produced by members of this group of organisms. Later investigators, especially Fildes, Knight and Pappenheimer [1933–35] made useful contributions on a broader scale, dealing mainly with growth studies. The most fundamental discovery, however, was made by Stickland [1934; 1935] who showed that a cell suspension of *Cl. sporogenes* is able to activate some amino-acids as hydrogen acceptors and others as hydrogen donators, thus enabling an intermolecular reaction between two amino-acids to occur, one being oxidized, the other reduced. This study of coupled reactions between pairs of amino-acids, brought about by a suspension of *Cl. sporogenes*, was continued and enlarged by Woods [1936].

It is striking that among the many naturally occurring amino-acids there is such a limited number that can be used either as hydrogen donators or acceptors. It has been shown, for instance, that tryptophan belongs to the "essential" amino-acids for the growth of *Cl. sporogenes*; but, so far as can be deduced from previous work, this amino-acid plays no role as acceptor or donator. The question is whether or not the "Stickland reaction" is the only reaction which provides *Cl. sporogenes* with the necessary energy. It has also been shown that bacteria belonging to the facultative anaerobic group are able to activate as hydrogen donators many biochemically interesting compounds, such as alcohol, formic acid, succinic acid, glucose etc. The work of Stickland, on the other hand, suggests that in the case of *Cl. sporogenes* there are certain amino-acids that can be activated as hydrogen donators (or acceptors), while non-amino-acids do not seem to play any important role in this respect. Nevertheless, *Cl. sporogenes* actively ferments certain carbohydrates and, since a correlation between fermentability of sugars and their suitability to act as powerful hydrogen donators is always found, one wonders why glucose is not a better hydrogen donator.

This paper presents information on the conditions under which various substrates act as hydrogen donators, and the influence of different factors involved, in the metabolism of the anaerobes, particularly *Cl. sporogenes*. It

appeared likely that a cultivation period of 40–45 hr. as used by Stickland, was too long for an organism which so readily goes into the spore stage. That the age of the bacterial culture may have a very great influence upon the activity of the enzymes was pointed out recently for *B. coli* by Wooldridge *et al.* [1936], and by Wooldridge & Glass [1937]. The *p*H of the culture medium during the growth of the bacteria, a factor which was not previously considered to any extent, appeared also to bear an important relation to the activity of the enzymes. The most important factor, however, which indicates the power of the micro-organism to activate certain substrates as hydrogen donators was found to be the *p*H at which the dehydrogenation experiment was conducted. Previously little attention was given to this factor, a *p*H of about 7 being usually chosen.

There may be additional unknown factors, consideration of which is required in order to obtain a clear insight into the enzyme activity of these micro-organisms.

## Experimental

The experiments described in this paper were carried out with washed suspensions of *Cl. sporogenes*. After it had been established that different pure cultures behaved similarly, the main work was done with a culture obtained from the American Type Culture Collection and labelled No. 319. Tubes containing 10 ml. of medium (3 % neopeptone solution at *p*H 7·4) were inoculated from the stock culture in brain medium. After 24 hr. incubation these tubes were used for the inoculation of the culture bottles.

Unless otherwise stated, the culture medium consisted of 250 ml. of a 3 % solution of neopeptone (Difco standardized) in distilled water adjusted with NaOH to *p*H 7·4 before sterilization. No change in *p*H takes place during the growth period. The flasks were kept in a McIntosh and Fildes anaerobic jar and incubated at 37° for a period of 16–20 hr., sufficient to insure a heavy growth of the organisms. The culture was then centrifuged and washed with 250 ml. of freshly boiled (oxygen-free) saline solution, and finally suspended in 10–20 ml. of 0·85 % NaCl solution. Microscopic examinations showed that the bacteria were nearly all in the vegetative stage, practically no spores being observed. Smears on nutrient agar plates showed that no contamination was present. The enzymic activity of such a suspension was exceedingly high. However, since it decreased very rapidly on storage, even under anaerobic conditions, the experiments were carried out immediately after preparing the suspension. The method used for the investigation of the various substrates as hydrogen donators was the Thunberg technique as modified by Keilin. Thus in a hollow stopper of about 2 ml. capacity was placed 1 ml. of the bacterial suspension. The following constituents were placed in the tube: 1 ml. of *M*/5 phosphate buffer at *p*H 7·4 (if not otherwise stated), 1 ml. of *M*/2000 Brilliant Cresyl Blue and 2 ml. of the substrate solution (usually *M*/10) adjusted with NaOH to *p*H 7·4. The final volume was 5 ml. so that the substrate concentration was *M*/50, unless otherwise stated. After thorough evacuation by an oil pump, the bacterial suspension in the hollow stopper was combined with the reaction mixture, incubated in a water bath at 40° and the time necessary for the reduction of the dye noted.

The first experiments were designed to test the behaviour of such suspensions of *Cl. sporogenes* towards the naturally occurring amino-acids, some synthetic amino-acids and also towards many substances of biological interest.

As may be seen from Table I, among the compounds studied there are some amino-acids which under the conditions of this experiment act as outstanding hydrogen donators, namely *d*-alanine, *l*-leucine, *l-iso*leucine, *dl*-α-amino-*N*-valeric acid, *d*-valine. Among them *dl*-α-amino-*N*-valeric acid is a synthetic

Table I. *Relative velocities of dehydrogenation of various substrates by suspensions of* Cl. sporogenes *(16–20 hr. culture in 3% neopeptone at* p*H 7·4) in presence of Brilliant Cresyl Blue at* p*H 7·4*

The velocity with *d*-alanine is taken as standard = 100

| 100–50 | | 10–1 | | 0 | |
|---|---|---|---|---|---|
| *d*-Alanine | 100 | Glycerol | 9 | Propionic acid | 0 |
| *l*-Leucine | 100 | *l*-Aspartic acid | 9 | Methyl alcohol | 0 |
| *l-iso*Leucine | 100 | *d*-Glutamic acid | 9 | Formaldehyde | 0 |
| *dl*-α-Amino-*N*-valeric acid | 100 | Malic acid | 9 | Glycine | 0 |
| *d*-Valine | 76 | Glycol | 8 | *l*-Proline | 0 |
| Pyruvic acid | 51 | Glyceric acid | 8 | *l*-Oxyproline | 0 |
| 50–25 | | *l*-Dihydroxyphenylalanine | 8 | Maleic acid | 0 |
| Allyl alcohol | 40 | Histamine | 8 | Glutaric acid | 0 |
| *l*-Histidine | 37 | Laevulose | 7 | Glycollic acid | 0 |
| *l*-Phenylalanine | 28 | Fumaric acid | 7 | Glyoxal | 0 |
| 25–10 | | Formic acid | 7 | β-Hydroxybutyric acid | 0 |
| Butylene glycol | 24 | *iso*Propyl alcohol | 6 | Acetic acid | 0 |
| Ethyl alcohol | 20 | *d*-Ornithine | 6 | *d*-Arginine | 0 |
| *n*-Butyl alcohol | 20 | Hydantoin | 4 | Acetamide | 0 |
| *l*-Tryptophan | 17 | *dl*-Tartaric acid | 4 | Acetylurea | 0 |
| *l*-Serine | 16 | Succinimide | 4 | Glycine anhydride | 0 |
| *l*-Tyrosine | 16 | Butyric acid | 4 | Oxaluric acid | 0 |
| *l*-Asparagine | 13 | Acetaldehyde | 4 | Urea | 0 |
| | | | | Barbituric acid | 0 |
| *d*-Glucose | 12 | Succinic acid | 3 | Creatinine | 0 |
| | | | | Creatine | 0 |
| 1 : 3-Butylene glycol | 12 | Dialuric acid | 3 | Acetoine | 0 |
| | | | | Formamide | 0 |
| *n*-Propyl alcohol | 12 | Citric acid | 3 | Alloxanthine | 0 |
| | | | | Alloxan | 0 |
| Lactic acid | 11 | *l*-Lysine | 3 | Betaine | 0 |
| | | | | Allantoin | 0 |
| Glyceric aldehyde | 10 | Dihydroxyacetone | 2 | Guanine | 0 |
| | | | | Guanidine | 0 |
| Malonic acid | 10 | Oxalic acid | 2 | | |

acid and is not a constituent of proteins. However, a great variety of non-amino-acids are also able to act as hydrogen donators. Especially is this the case with pyruvic acid and different alcohols whereas glucose, lactic acid and glyceric aldehyde are fairly good donators.

Comparing our results with those of Stickland, who investigated only a very limited number of substrates, there is fairly good agreement, although our numbers for most of the substrates are somewhat higher. We believe that these differences are due to the fact that the vitality of our suspensions was higher than that of the suspensions used by Stickland, who cultivated the bacteria on a tryptic digest of caseinogen for 40–45 hr. It is possible also that the *p*H (not given in his paper) of Stickland's culture medium differed from ours. It has been our experience that very young bacterial cultures (16 hr.) are able to attack the compounds under study much more readily than older cultures, which often fail to activate some of the substrates as hydrogen donators. As has been pointed out, there are other factors that are of importance in influencing the dehydrogenase reactions which will be discussed in more detail.

### (*a*) *Influence of* p*H on dehydrogenation*

Since it was impossible to investigate all of the compounds that may act as hydrogen donators, this work was restricted to four typical compounds: an amino-acid (*d*-alanine), a keto-acid (pyruvic acid), an alcohol (ethyl alcohol) and an aldehyde (acetaldehyde).

The buffer solutions used for these experiments were prepared from solutions of $M/5$ $KH_2PO_4$, $M/5$ $Na_2HPO_4$ and $M/5$ NaOH. Other buffer mixtures (phthalate, borate, citrate, veronal) were found unsatisfactory, partly because they inhibit dehydrogenase activity and partly because they do not cover the entire desired *p*H range. The buffering capacity of the phosphate buffer is almost zero in the more alkaline region but, owing to the fact that the theoretically possible amount of decomposition products is extremely small (equivalent to the amount of dye added), it was found that no change in the *p*H occurred during the experiment, even in this poorly buffered region. For each substrate the *p*H giving the most rapid reduction of the dye was taken as 100 (=*p*H optimum). (Figs. 1–4, solid lines.)

As may be seen from these figures, the effect of *p*H on the rate of dehydrogenation is different for each of the four substrates investigated. The dehydrogenase action with *d*-alanine has its optimum at *p*H 9·3, with ethyl alcohol at *p*H 8·5, with acetaldehyde at *p*H 6·7 and with pyruvic acid at *p*H 6·0.

The course of the *p*H curves is in most cases a rather irregular one. This may be due to the fact that the dehydrogenation reaction is not a simple one, but occurs in different stages, the product of the first reaction being further converted by intermediate processes.

The sharp declines of the curves obtained, especially for pyruvic acid and acetaldehyde in the acid region, may be due to the fact that longer reduction times cause a marked destruction of the enzymes in this region. If this be true, the curves show (1) the resultants of *p*H-enzyme activity and *p*H-enzyme destruction, or (2) that the dye used at these *p*H values is thermodynamically insufficient with respect to the substrates or substrate-decomposition products.

These curves show clearly that it is not correct to conclude that an amino-acid, as for instance *d*-alanine, is a better hydrogen donator than, for example, pyruvic acid, as Table I suggests, since at another *p*H (6·0) the reverse order is shown. The degree of activity depends upon the *p*H.

The *p*H curve for the dehydrogenation of pyruvic acid is interesting from another point of view, namely it was found that *Cl. sporogenes* is able to ferment this compound with $CO_2$ formation under anaerobic conditions without the presence of any dye, also with an optimum at *p*H 6·0. (To be published later.)

In order to determine the influence of *p*H during the bacterial growth on dehydrogenation the experiments were repeated exactly as above mentioned, but instead of cultivating the bacteria at *p*H 7·4, it was done at *p*H 6·4, on the same medium. During the growth the *p*H increased slightly to *p*H 6·5 at the end of the culture period (20 hr.). The same *p*H curves for dehydrogenase activity could no longer be obtained. All curves showed a shift towards the acid range (Figs. 1–3, dotted lines), which was extremely pronounced in the case of *d*-alanine. Here the second optimum (in the former curve at *p*H 7·8) had shifted to *p*H 7·0 and became the main optimum, while the main optimum in the former curve (at *p*H 9·3) had shifted towards *p*H 8·2 and had become a secondary optimum. The result with pyruvic acid was similar, but the deviations for ethyl alcohol were not so great. This means that by simply cultivating the bacteria in a more acid range on the same medium, the *p*H-optima for the different substrates were shifted more or less. The explanation of this phenomenon, for which no analogy is found in the literature, is hypothetical at present, namely that the bacteria quickly adapt themselves to the more acid conditions in the culture medium by synthesizing slightly different enzymes which are more able to attack the various substrates at a lower *p*H.

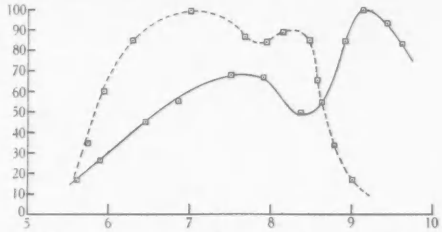


Fig. 1. *Cl. sporogenes.* *p*H-optimum for *d*-alanine dehydrogenase. *p*H of the culture medium 7·4 = ————; *p*H of the culture medium 6·4 = – – – –. Abscissa: *p*H. Ordinate: velocity.

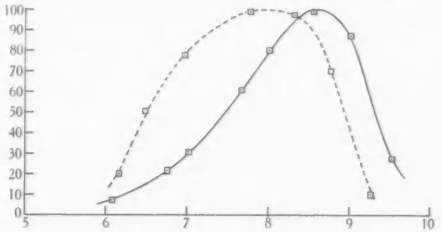


Fig. 2. *Cl. sporogenes.* *p*H-optimum for ethyl alcohol dehydrogenase. *p*H of the culture medium 7·4 = ————; *p*H of the culture medium 6·4 = – – – –. Abscissa: *p*H. Ordinate: velocity.

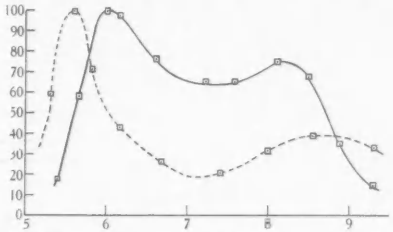


Fig. 3. *Cl. sporogenes.* *p*H-optimum for pyruvic acid dehydrogenase. *p*H of the culture medium 7·4 = ————; *p*H of the culture medium 6·4 = – – – –. Abscissa: *p*H. Ordinate: velocity.

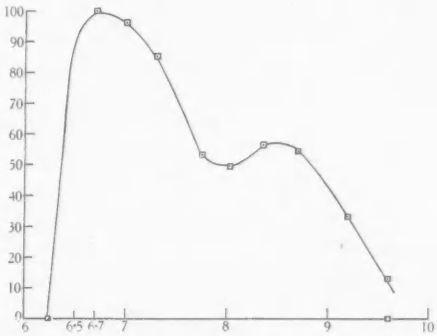


Fig. 4. *Cl. sporogenes.* *p*H-optimum for acetaldehyde dehydrogenase. *p*H of the culture medium 7·4. Abscissa: *p*H. Ordinate: velocity.

Whether this phenomenon is universal for the genus *Clostridium* is unknown at present, although similar results were obtained with another representative, *Cl. histolyticum*.

Table II. *Shift of the* p*H-optima for the dehydrogenation of* d*-alanine, pyruvic acid and ethyl alcohol by suspensions of* Cl. sporogenes *and* Cl. histolyticum *cultivated on the same medium (3% neopeptone), but at different* p*H*

| *p*H-optimum for dehydrogenation of | *Cl. sporogenes* cultivated at | | *Cl. histolyticum* cultivated at | |
|---|---|---|---|---|
| | *p*H 6·4 | *p*H 7·4 | *p*H 6·4 | *p*H 7·4 |
| *d*-Alanine | 7·0 | 9·3 | 7·8 | 9·3 |
| Pyruvic acid | 5·6 | 6·0 | 5·4 | 6·0 |
| Ethyl alcohol | 8·0 | 8·5 | 8·3 | 8·3 |

(*b*) *The influence of the medium*

In order to see whether the composition of the medium on which *Cl. sporogenes* was grown had any influence upon the dehydrogenation activity, three different media were prepared: (1) the usual 3% neopeptone, (2) a tryptic digest of 3% gelatin, (3) a tryptic digest of 3% casein, all adjusted to *p*H 7·4. This *p*H did not alter during the cultivation time. The bacterial suspensions were made in the usual way and their activities compared against eight different substrates at *p*H 7·4. No significant difference was found, which justifies the conclusion (at least as far as the substrates studied are concerned) that the source of protein on which *Cl. sporogenes* is cultivated has little if any influence on the dehydrogenases produced. As the results obtained with the different media are practically identical, the figures for any one may be seen by referring to Table III, first column.

Table III. *Activity of suspensions of* Cl. sporogenes *(4·2 × 10⁹ cells per ml.) cultivated for 20, 45 and 70 hr. on 3% neopeptone* (p*H 7·4*), *measured on eight different substances at* p*H 7·4*

The velocity with *d*-alanine is taken as standard = 100. (Reduction time is given in min.)

| | Age of the culture | | | | | |
|---|---|---|---|---|---|---|
| | 20 hr. | | 45 hr. | | 70 hr. | |
| Substrates | Reduction time | Velocity | Reduction time | Velocity | Reduction time | Velocity |
| Glucose | 25·5 | 12 | 51 | 10 | — | — |
| Formic acid | 43 | 7 | — | — | — | — |
| *l*-Tryptophan | 17·5 | 17 | 33 | 15 | 100 | 9 |
| *l*-Asparagine | 21·3 | 14 | 23 | 22 | 58 | 15·5 |
| *d*-Alanine | 3 | 100 | 5 | 100 | 9 | 100 |
| *d*-Glutamic acid | 30·3 | 9 | 48 | 10 | 115 | 8 |
| Pyruvic acid | 5·7 | 51 | 12 | 42 | 22·5 | 40 |
| Ethyl alcohol | 15 | 20 | 9 | 56 | 9 | 100 |

(*c*) *Influence of the age of the culture*

In order to investigate the influence of the age of the culture on dehydrogenase activity of the suspension obtained from such culture, a bottle with 750 ml. of 3% neopeptone was inoculated. During the incubation period of 70 hr. equal proportions were removed for study at 20, 45 and 70 hr. Cell suspensions were prepared and in all cases diluted until the suspension contained $4·2 \times 10^9$ cells per ml.

As may be seen from Table III, the activity of the bacterial suspensions towards many of the substrates decreases considerably with increasing age of the culture; the younger the culture, the greater the activity. A culture of 70 hr. gives a bacterial suspension which has a reduction time for *d*-alanine three times that of the 20 hr. culture. Similar values were obtained for *l*-asparagine and *d*-glutamic acid, while for pyruvic acid these numbers are four times as great and for *l*-tryptophan six times as great. Formic acid and glucose were not dehydrogenated at all by the 70 hr. culture. The behaviour towards ethyl alcohol was in decided contrast to that shown towards glucose. The bacteria from the 70 hr. culture dehydrogenated this substrate much more actively than those from the 20 hr. culture. With the organisms from the 70 hr. culture ethyl alcohol compares favourably with *d*-alanine as a hydrogen donator, while with the organisms from the 20 hr. culture it was only one-fifth as efficient.

### (*d*) *Influence of addition of glucose to culture medium*

The experiments were carried out in the same manner as the preceding one, except that 1 % of glucose was added to the 3 % neopeptone medium. In order to avoid marked changes in the *p*H during the growth due to sugar fermentation, an initial *p*H of 6·1 was chosen for the experiment. This *p*H remained fairly constant during the whole incubation time, during which considerable gas was produced. Unfortunately, therefore, the results shown in Tables III and IV are not directly comparable. An attempt was made, without much success, to cultivate *Cl. sporogenes* on neopeptone at *p*H 6·1 in order to obtain comparable results. The *p*H of the solution gradually increased until after 1–2 days it had reached *p*H 7·0 (Table IV).

Table IV. *Activities of suspensions of* Cl. sporogenes *(4·5 × 10⁹ cells per ml.) cultivated for 20, 45 and 70 hr. on 3 % neopeptone—1 % glucose at* p*H 6·1, measured on 9 different substrates at* p*H 7·4*

| | Age of the culture | | | | | |
|---|---|---|---|---|---|---|
| | 20 hr. | | 45 hr. | | 70 hr. | |
| Substrates | Reduction time | Velocity | Reduction time | Velocity | Reduction time | Velocity |
| Glucose | 3·5 | 33 | 5 | 38 | 8 | 38 |
| Formic acid | 20·7 | 4·5 | — | — | — | — |
| *l*-Tryptophan | 10 | 9 | 22 | 6 | 52·5 | 6 |
| *l*-Asparagine | 7·3 | 14 | 20 | 7 | 27 | 11 |
| *d*-Alanine | 1·2 | 100 | 2 | 100 | 3 | 100 |
| *d*-Glutamic acid | 9·7 | 10 | 37 | 2 | 51·5 | 6 |
| Pyruvic acid | 2·2 | 55 | 3·5 | 56 | 4·7 | 63 |
| Ethyl alcohol | 0·7 | 168 | 3 | 67 | 15 | 20 |
| Lactic acid | 14·5 | 5·5 | 26·5 | 4 | 63 | 5 |

A glance at Table IV shows at once that glucose now acts as a powerful hydrogen donator. Even after 70 hr. cultivation its activity is exceedingly strong. The behaviours of formic acid, *l*-tryptophan, *l*-asparagine, *d*-alanine and *d*-glutamic acid are similar to those shown in Table III. Pyruvic acid dehydrogenation is increased gradually by longer incubation when glucose is present in the medium, while Table III shows a gradual decrease in activity when the medium does not contain glucose.

Most striking, however, is the behaviour of the alcohol dehydrogenation. After 20 hr. incubation time the activity of the alcohol dehydrogenase is extremely high and exceeds that of *d*-alanine, which has always been the most active

hydrogen donator, by even more than 50%. The activity of alcohol dehydrogenase decreases rapidly, however, as may be seen from the values obtained with the cultures of 45 and 70 hr. This is in direct contrast to the increasing activity during ageing when glucose is not added to the culture medium (Table III).

Any attempt to explain this phenomenon would be purely speculative. Our knowledge of catabolism in anaerobes is too restricted at present to offer any satisfactory explanations.

### (e) *Influence of substrate concentration*

We investigated the behaviour of washed *Cl. sporogenes* suspensions towards the substrates *d*-alanine, pyruvic acid and ethyl alcohol, in dilutions varying from *M*/5 to *M*/50,000. The results given in Fig. 5 show the behaviour as related to substrate concentration. The concentration of *d*-alanine within the limits of *M*/5–*M*/200 is without influence upon the reduction time of the dye, indicating a relatively high substrate affinity, while pyruvic acid and ethyl alcohol alike show a quite different behaviour, being greatly influenced by the substrate concentration. The experiments were carried out at the *p*H optima for the investigated compounds.

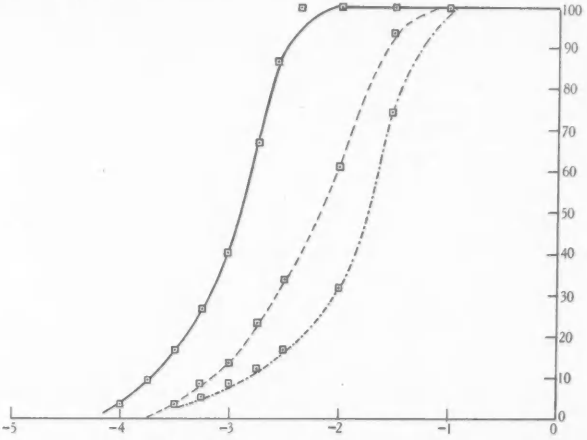


Fig. 5. *Cl. sporogenes.* Influence of the substrate concentration. Substrates: *d*-alanine = ————; pyruvic acid = – – – –; ethyl alcohol = –·–·–·–. Abscissa: mol. substrate concentration log −1 ... −5. Ordinate: velocity 0–100.

### (f) *Influence of addition of poisons on dehydrogenation*

The influence of $As_2O_3$ and HCN in different concentrations on the dehydrogenation of *d*-alanine, pyruvic acid and ethyl alcohol as substrates (*M*/50) was studied.

The concentrations of HCN[1] and $As_2O_3$ used were *M*/50–*M*/5000 expressed for the total volume.

[1] The investigations with the addition of the volatile HCN had to be carried out cautiously to avoid as far as possible an escape of HCN during the evacuation of the Thunberg tubes. For this purpose the HCN was placed in the hollow stopper at a more alkaline reaction and the buffer used adjusted in such way that, after evacuating and combining the reaction mixtures in the tubes, the required *p*H for the determination was obtained.

It may be seen from Fig. 6 that the behaviours of both $As_2O_3$ and HCN are similar toward one substrate, but that these behaviours may be completely different for different substrates. The dehydrogenation of ethyl alcohol is inhibited by increasing concentrations of HCN or $As_2O_3$, the dehydrogenation of *d*-alanine on the other hand is activated by addition of these compounds in concentrations up to $M/50$. This latter fact is not in agreement with the observations made by Stickland, who found that these compounds inhibited completely the dehydrogenation of *d*-alanine. By the addition of arsanilic acid we were also able to obtain an activation of the *d*-alanine dehydrogenation. The dehydrogenation of pyruvic acid is not affected by the addition of HCN, while arsenious acid, independently of the concentration, inhibits to the extent of 14 %.

Addition of $CuSO_4$ even in concentrations as low as $5 \times 10^{-4}\ M$ inhibits completely all dehydrogenation reactions studied on the same substrates, while on the other hand addition of NaF, even in concentrations as high as $M/5$, is without any influence upon the reduction of the dehydrogenases studied.

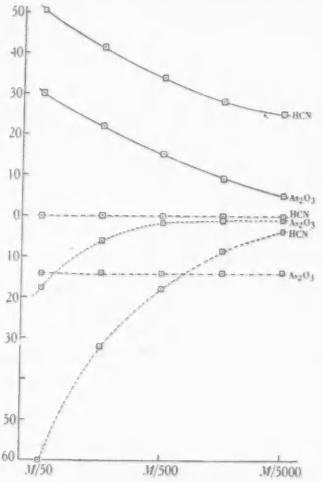


Fig. 6.

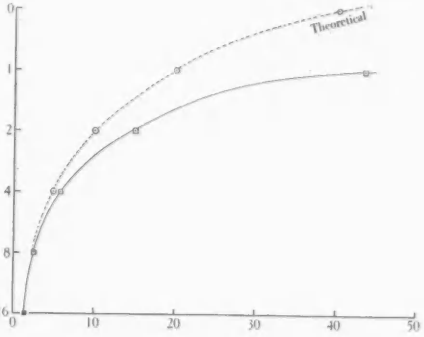


Fig. 7.

Fig. 6. *Cl. sporogenes.* Influence of HCN, $As_2O_3$ upon *d*-alanine, pyruvic acid, ethyl alcohol dehydrogenases. *d*-alanine = ————; pyruvic acid = – – – –; ethyl alcohol = –·–·–· (to the lines has to be added HCN, resp. $As_2O_3$). Abscissa: log. mol. concentration of HCN resp. $As_2O_3$. $M/50$–$M/5000$. The substrate concentration is constant = $M/50$. Ordinate: 0–50 = % activation; 0–60 = % inhibition.

Fig. 7. *Cl. sporogenes.* Influence of the bacterial concentration. Substrate *d*-alanine ($M/50$). Abscissa: reduction time in min. 1–50. Ordinate: ml. bacteria suspension 16–1.

### (*g*) *Influence of variation in enzyme concentration*

The effect of decreasing concentrations of washed suspensions of *Cl. sporogenes* used for dehydrogenation reactions was studied on the substrate *d*-alanine. A thick suspension of bacteria in saline was prepared in the usual way and diluted in geometric progression. Substrate and dye concentrations were in all cases the same, saline being added where necessary to make a total volume of 5 ml.

It was found that there was by no means a constant ratio between reduction time and bacterial concentration in the entire range of the dilutions. An approximately constant ratio exists only when rather high concentrations of bacteria are employed. The lower the bacterial concentration, the more pronounced the deviation from the hyperbolic function (Fig. 7).

Probably this is due to the fact that during the manipulations in which a limited contact with air is difficult to avoid, a small part of the activity is lost. As a matter of fact, smaller amounts of the suspensions or dilute suspensions are less protected against oxygen and a loss in activity is the consequence.

## Discussion

These investigations show that there are a great many factors which influence very markedly the results of dehydrogenation reactions with washed suspensions of *Cl. sporogenes* when reversibly reducible dyes are used as hydrogen acceptors. The following are important factors.

(1) The age of the bacterial culture. Usually the activity of the bacterial culture decreases with increasing incubation time. This is by no means always the case. Some substrates, such as ethyl alcohol, are more rapidly dehydrogenated by older cultures, when cultivated on neopeptone at *p*H 7·4.

(2) The *p*H of the medium during the growth of the bacteria. Marked changes in the *p*H curves for the different substrates were observed, when the bacteria were cultivated in a more acid medium. In general, these curves show that the substrates are attacked more readily in the acid ranges.

(3) The composition of the culture medium. Addition of glucose to the culture medium not only increased the ability of the organisms to use glucose as a hydrogen donator but also ethyl alcohol to a marked degree.

These factors, related to the conditions under which the bacteria have been cultivated, indicate the ease with which the organisms adapt themselves to changes in their environment. Organisms grown in broth only are not identical with those grown in broth to which glucose has been added, as the latter have developed to a high degree the enzyme mechanism necessary to utilize glucose, while this mechanism is rudimentary in organisms grown in broth alone. Repeated cultivation in a glucose-containing medium gradually develops the glucose-utilizing mechanism.

When cultivated in media of low *p*H the organisms adapt their enzyme production mechanism so that the substrates are more readily attacked at such *p*H values. It was impossible to demonstrate this for higher *p*H values. Conditions in old and young cultures differ. The changes in activity observed in this case are probably related to changes in the medium. The complexity of such medium makes it impossible to understand the reason for the different activities.

It is highly probable that this phenomenon of adaptability is not restricted to *Cl. sporogenes* but will be found in the entire microflora. Wooldridge recently described the influence on dehydrogenation reactions of ageing of a culture of *Bact coli*. It may be recalled that some lactic acid bacteria and yeasts are able to ferment certain carbohydrates only when they have been cultivated in the presence of these carbohydrates.

The present study shows that, in addition to the factors related to the conditions under which the bacteria are cultivated, there are also factors of importance in connexion with the preparation and investigation of the suspensions.

"Repeated washings" have been generally advocated in the preparation of bacterial suspensions for dehydrogenation experiments. It seems that with such procedure there is danger that the sensitive dehydrogenases, especially, may be partially inactivated or destroyed before the experiment is begun. When the culture is centrifuged in conical beakers at high speed a sharp separation of sediment and medium is obtained, and in this case one washing is sufficient to obtain a suspension with a very high activity and a very low blank value.

To locate definitely the dehydrogenating properties of the organisms, it was attempted to prepare an "enzyme solution" (after finding that the dehydrogenases are not secreted in the culture medium during the growth) by a careful destruction of the cell structure. However, this was not successful. The extreme lability of the dehydrogenases of *Cl. sporogenes*, even under anaerobic conditions at 0°, is remarkable and in striking contrast to that of *Bact. coli*, a suspension of which may be kept for a considerable time in the ice-box without loss of activity.

The irregularity of the *p*H curves in Figs. 1–4 make them appear questionable. That the irregularities are real was demonstrated by repeating the experiments several times. This fact may be ascribed to the presence of other products, which arise from the original substrate. In the case of pyruvic acid for example, it was found that, when this substrate is incubated without dye under anaerobic conditions with a suspension of *Cl. sporogenes*, a rapid fermentation takes place as a result of which 1 mol. of $CO_2$ is formed per mol. pyruvic acid, indicating the formation of acetaldehyde. There are further indications that the acetaldehyde formed is converted into ethyl alcohol and acetic acid. This means that, when Brilliant Cresyl Blue also is present, not only pyruvic acid may be dehydrogenated but also acetaldehyde and ethyl alcohol, the fermentation products of pyruvic acid. These different substrates are perhaps decomposed by different enzymes at different *p*H-optima, the *p*H curve representing a resultant of all these processes. Similar phenomena may occur in the breakdown of *d*-alanine and other more complicated compounds.

Whether there is one enzyme (an "oxidoreductase") responsible for the dehydrogenation of the various substrates or whether there are produced dehydrogenases specific for each substrate or groups of substrates composed of similar compounds is not known. The different behaviours towards HCN and $As_2O_3$ suggest the latter possibility.

Finally, one important point should be stressed. The experiments show that *Cl. sporogenes* under certain conditions can activate not only amino-acids, as has been found hitherto but also many other compounds. When the organisms are grown on glucose broth, ethyl alcohol is an even better hydrogen donator than *d*-alanine. This suggests the possibility that a "Stickland reaction" may occur for instance between an amino-acid and alcohol.

As will be shown in a later publication there exist also non-amino-acids which act as hydrogen acceptors, suggesting the possibility of a reaction between *d*-alanine as donator and, as an example, acetylmethylcarbinol as acceptor. Even reactions between non-amino-acids should be possible with suspensions of *Cl. sporogenes*. The "Stickland reaction" would then have a more general significance if these reactions were possible.

We hope to continue investigations in this direction.

## Summary

1. A great number of amino-acids and biologically interesting compounds have been investigated as to their ability to act as hydrogen donators with washed suspensions of *Cl. sporogenes* under the stated conditions. It has been found

that many of these compounds could be dehydrogenated, although usually certain amino-acids are better hydrogen donators.

2. A study was made of the conditions which may influence the results: the age of the culture; the *p*H of the medium; the composition of the medium; and the *p*H at which the dehydrogenations were performed.

3. Usually the activity towards different substrates decreases during continued incubation. Some substrates like ethyl alcohol, however, are more rapidly dehydrogenated by older cultures when cultivated on neopeptone.

4. The *p*H curves for different substrates are completely different and the *p*H optima for dehydrogenation may vary greatly. Cultivated on neopeptone at *p*H 7·4 the *p*H-optimum for *d*-alanine was found to lie at 9·3, for ethyl alcohol at 8·5, for acetaldehyde at 6·7, and for pyruvic acid at 6·0. This indicates that comparison of dehydrogenating potencies must be made with due regard to the *p*H at which they have been investigated.

5. Marked changes in the *p*H curves for the different substrates were observed when the bacteria employed were cultivated at a more acid reaction than *p*H 7·4. In general these curves showed a shifting towards the acid side, indicating an adaptation of the organism, so that these substrates could be attacked more easily at low *p*H.

6. Addition of glucose to the culture medium not only increased the ability of the organism to use glucose as a hydrogen donator, but also, more markedly, that of ethyl alcohol.

7. The different behaviours of the dehydrogenases acting on *d*-alanine, pyruvic acid or ethyl alcohol towards poisons such as HCN, $As_2O_3$, NaF, $CuSO_4$ are described.

The authors wish to express their thanks to Dr Ellice McDonald, Director, for his interest and support throughout this work.

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## ADDENDUM

While this paper was in the press a review, by Yudkin, appeared in *Biological Reviews*, **13**, 1 [1938], critically summarizing all the known cases of "adaptability" or "training" of micro-organisms. This will be discussed in a subsequent paper.

# LVII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS

## LVI. LUTEOLEERSIN AND ALBOLEERSIN; METABOLIC PRODUCTS OF *HELMINTHOSPORIUM LEERSII* ATKINSON. A POSSIBLE OXIDATION-REDUCTION SYSTEM

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In a series of investigations carried out in collaboration with Prof. R. Robinson of the University of Oxford, a number of crystalline metabolic products, isolated from the mycelium of different species of moulds in the genus *Helminthosporium*, have been isolated. These include four hitherto undescribed hydroxyanthraquinones: (*a*) helminthosporin (2-methyl-4:5:8-trihydroxyanthraquinone) from *H. gramineum* Rabenhorst and *H. catenarium* Drechsler [Charles *et al.* 1933; Raistrick *et al.* 1934]; (*b*) catenarin (a $\beta$-(hydroxymethyl)-1:5:8-trihydroxyanthraquinone) from *H. catenarium*, *H. gramineum* and *H. velutinum* Link [Raistrick *et al.* 1934]; (*c*) cynodontin (2-methyl-1:4:5:8-tetrahydroxyanthraquinone) from *H. cynodontis* Marignoni and *H. euchlaenae* Zimmermann [Raistrick *et al.* 1933] and (*d*) tritisporin (6(or 7)-hydroxymethyl-1:3:5:8-tetrahydroxyanthraquinone) from *H. tritici-vulgaris* Nisikado [Raistrick *et al.* 1934]. More recently a substituted xanthone, ravenelin (3-methyl-1:4:8-trihydroxyxanthone) was isolated in a crystalline form from cultures of *H. Ravenelii* Curtis and *H. turcicum* Passerini [Raistrick *et al.* 1936].

It has now been found that the mycelium of *H. leersii* Atkinson contains considerable amounts of two different, but chemically closely related, metabolic products which have not been described previously and for them the names *luteoleersin* and *alboleersin* are proposed.

The fungus *H. leersii* was first reported by Atkinson [1897], who isolated it from the leaves of *Leersia virginica* Willd. (=*Homolocenchrus virginicus* (Willd.)) Britton at Auburn, Alabama in September 1891. It is widely distributed in America and was found by Drechsler [1923] as a parasite on the leaf-blades of *L. virginica* in Connecticut in 1920 and in Washington in 1921. It forms dark spots on the leaves. When grown on artificial media it develops a dense grey aerial mycelium.

Luteoleersin, $C_{26}H_{38}O_7$, m.p. 135°, $[\alpha]^{18°}_{5461}+214°$ ($c=0{\cdot}456$ in alcohol) was obtained as beautiful yellow rods or blunt-ended needles; alboleersin, $C_{26}H_{40}O_7$, m.p. 215–216°, $[\alpha]^{18°}_{5461}+274°$ ($c=0{\cdot}430$ in alcohol) as long, colourless needles. Luteoleersin contains two active hydrogen atoms and alboleersin three. The empirical formulae for both substances are advanced tentatively at present, since, although they are based on a large number of C and H determinations and on mol. wt. estimations, it has not been found possible to confirm them by the analysis of derivatives as no crystalline derivatives have in fact been isolated.

It is hoped to obtain further evidence for these empirical formulae from further work which is in progress on these substances and will be reported later.

The biochemical significance of luteoleersin and alboleersin lies we believe in their relationship to each other. They are very readily interconvertible *in vitro*, and while both substances have been isolated from all cultures of *Helminthosporium leersii* examined, their relative proportions have varied in different preparations. Luteoleersin may be readily reduced to alboleersin by catalytic reduction with palladium-charcoal-hydrogen, or by heating with phenylhydrazine. Alboleersin may be smoothly and almost quantitatively oxidized to luteoleersin by treatment in the cold with $FeCl_3$. Luteoleersin behaves as a quinone or semiquinone and alboleersin as the corresponding phenol. It is believed that these two substances offer an example of an oxidation-reduction system in the life processes of the mould *H. leersii*. Several other examples have recently been encountered in these laboratories and will be reported at a later date, and one similar case was suggested by Birkinshaw & Raistrick [1932] in the two metabolic products given by both *Penicillium puberulum* Bainier and *P. aurantio-virens* Biourge, i.e. puberulic acid $C_8H_6O_6$, and the unnamed acid $C_8H_4O_6$, which were shown by Barger & Dorrer [1934] to be interconvertible.

## Experimental

### *History of the culture used*

The culture of *Helminthosporium leersii* Atkinson used throughout this work was purchased from the Centraalbureau voor Schimmelcultures, Baarn, Holland in June 1933. It was received by them from Nisikado who isolated it from diseased leaf blades of *Leersia virginica*. It bears the L.S.H.T.M. catalogue number Ag 132.

### *Cultural conditions*

Batches of 100 flasks of Czapek-Dox solution (glucose, 50 g.; $NaNO_3$, 2·0 g.; $KH_2PO_4$, 1·0 g.; KCl, 0·5 g.; $MgSO_4,7H_2O$, 0·5 g.; $FeSO_4,7H_2O$, 0·01 g.; distilled water, 1 l.), 350 ml. in each litre conical flask, were sown with a suspension of a culture of *Helminthosporium leersii*, Ag. 132, and incubated in the dark at 24°. The rate of growth was slow and incubation was continued for 90 days at which time the glucose had almost completely disappeared. All the flasks presented an even appearance. The mycelium was dark grey in colour, with a black margin and a black reverse. The filtered metabolism solution was peach-coloured. The mycelium was separated by filtration, washed with cold water, pressed, dried in a vacuum oven at 45–50° and weighed. (Wt. of mycelium, from 100 flasks in 3 representative experiments, 299 g.; 316 g.; 323 g.)

### *Extraction of alboleersin and luteoleersin*

The dried mycelium was ground in a coffee mill and thoroughly extracted for 3–4 working days in a Soxhlet apparatus with light petroleum, B.P. 40–50°, giving a yellow solution. At the end of each day the extraction flask was placed in the cold room and replaced the following day by a new flask containing fresh solvent. There separated from the light petroleum extracts on standing a greyish white product consisting essentially of crude alboleersin, the M.P. of which was usually 195–205°. The mother liquors from the crude alboleersin were combined, evaporated to a small volume and kept. There thus separated crude luteoleersin as a bright yellow crystalline powder, the M.P. of which, while very

variable, was between 120 and 135°. Considerable quantities of crude fat remained after evaporation to dryness of the final light petroleum mother liquors. The yields of crude alboleersin obtained in 3 representative experiments, using 100 flasks in each case, were 0·58, 1·95 and 1·55 g., while the corresponding yields of crude luteoleersin were 6·80, 4·39 and 5·46 g.

After exhaustive extraction with light petroleum, the mycelium was extracted with ether, giving considerable amounts of a dark brown extract, and then with $CHCl_3$ which gave smaller amounts of a dark green extract. No crystalline substances have, at present, been isolated either from the ether or $CHCl_3$ extracts.

*Purification of the metabolic products*

(*a*) *Alboleersin.* Some of the fractions of crude alboleersin were very impure. The different fractions, together with small amounts obtained in purifying the crude luteoleersin, were twice crystallized from benzene (charcoal). The purified alboleersin was thus obtained as practically colourless thin, pearly plates, M.P. 208–209° (decomp.). This product was then repeatedly crystallized from 65–70 % methyl alcohol (aqueous, by volume) to give clusters of long colourless, silky needles with a constant M.P. of 215–216°. The average yield of purified alboleersin obtained per 100 flasks was about 1 g.

(*b*) *Luteoleersin.* The crude luteoleersin was dissolved in a minimum volume of boiling light petroleum (B.P. 80–100°) and was separated from undissolved alboleersin. The luteoleersin which crystallized out on cooling the filtrate was purified to constant M.P. by recrystallization from light petroleum (B.P. 60–80°) and charcoal. The substance was finally obtained as beautiful yellow rods or blunt-ended needles, M.P. 135°. The average yield of purified luteoleersin obtained per 100 flasks was rather more than 2 g.

*Analysis and general properties of alboleersin and luteoleersin*

A number of different samples of both substances, dried to constant weight at 80–90° in a high vacuum, were analysed in duplicate by two different analysts with the following results.

(*a*) Alboleersin. Four samples analysed by Weiler: C, 67·44 % (average of 8 analyses—limits 67·25–67·72 %); H, 8·54 % (average of 8 analyses—limits 8·25–8·80 %). Two samples analysed by Schoeller: C, 67·00 % (average of 4 analyses—limits 66·86–67·10 %); H, 8·54 % (average of 4 analyses—limits 8·48, 8·65 %). $C_{26}H_{40}O_7$ requires C, 67·20; H, 8·68 %.

(*b*) Luteoleersin. Two samples analysed by Weiler: C, 67·45 % (average of 4 analyses—limits 67·35–67·60 %); H, 8·23 % (average of 4 analyses—limits 8·19–8·28 %). Duplicate portions of the same two samples analysed by Schoeller: C, 67·54 % (average of 4 analyses—limits 67·44–67·66 %); H, 8·18 % (average of 4 analyses—limits 8·00–8·35 %). $C_{26}H_{38}O_7$ requires C, 67·50; H, 8·28 %.

Neither substance contains N, S, P, Cl or methoxyl.

*Mol. wt.* (Dr A. E. Oxford); cryoscopic in dioxane. (*a*) Alboleersin. Found 389. $C_{26}H_{40}O_7$ requires 464. This estimation has probably given too low a result since the solution, which was colourless at the start, had become bright yellow at the end of the experiment. This is probably due to oxidation, since alboleersin when in solution is rather quickly oxidized to luteoleersin.

(*b*) Luteoleersin. Found 449. $C_{26}H_{38}O_7$ requires 462.

*Active hydrogen estimations* (Roth). (*a*) Alboleersin gave in pyridine 2·87 active H atoms per molecule at 21° and 3·00 at 95° calculated on the formula $C_{26}H_{40}O_7$.

(*b*) Luteoleersin gave in pyridine 2·0 active H atoms per molecule at 21° and 2·0 at 95° calculated on the formula $C_{26}H_{38}O_7$.

*Optical rotations.* (*a*) Alboleersin. $[\alpha]^{18°}_{5461}+274°$, $[\alpha]^{18°}_{5790}+242°$ ($c=0·4304$ in alcohol).

(*b*) Luteoleersin. $[\alpha]^{18°}_{5461}+214°$, $[\alpha]^{18°}_{5790}+232°$ ($c=0·4560$ in alcohol).

The abnormal fact that the specific rotation of luteoleersin is greater for the mercury yellow line (5790) than for the mercury green line (5461) was checked by independent observers.

Alboleersin and luteoleersin are fairly soluble in most organic solvents but are almost insoluble in water. Luteoleersin is in general more soluble than alboleersin. Alboleersin may be readily crystallized from benzene, 50% aqueous alcohol, or, better, from 65–70% aqueous methyl alcohol, and luteoleersin from light petroleum, B.P. 60–80°.

On heating in air (e.g. in a M.P. determination) alboleersin begins to darken and decompose at about 150–160° and finally melts to a dark red liquid at 215–216° (decomp.). Alboleersin behaves in a characteristic way on heating in a high vacuum at 90°. The colourless crystals turn a beautiful mauve colour which remains stable overnight *in vacuo* but the crystals again become colourless on short exposure to air. No detectable change in weight occurred during this colour change. Alboleersin slowly turns yellow on continued exposure to air, probably due to oxidation to luteoleersin. Luteoleersin melts smoothly at 135° and is quite stable in air.

*Reactions of alboleersin and luteoleersin*

Alboleersin dissolves in cold conc. $H_2SO_4$ to give a pale yellow colour which changes to dark brown on standing overnight. With the same reagent luteoleersin gives a pale blue solution changing quickly to green and then to a dirty yellowish green.

An alcoholic solution of alboleersin gives with $FeCl_3$ a bright yellow colour. Luteoleersin gives no reaction.

With aqueous NaOH alboleersin slowly dissolves to give a soapy yellow solution, changing quickly in colour to pinkish mauve and on standing becoming nearly colourless. Luteoleersin is insoluble in aqueous NaOH. With alcoholic NaOH a characteristic purple colour is developed after a few seconds.

Luteoleersin is readily reduced in alcoholic solution with Zn and HCl to give a colourless solution.

Both substances instantly reduce $KMnO_4$ in acetone, and neither of them gives a sterol reaction.

Luteoleersin gives the reaction, characteristic of quinones, with substances containing an active methylene group. Thus, on adding to a very dilute solution of luteoleersin in 50% pyridine one drop of ethyl acetoacetate together with a few drops of 2*N* NaOH, a beautiful pale blue coloration is obtained. If the ethyl acetoacetate is omitted from this test, only the characteristic purple coloration is produced.

*Conversion of luteoleersin into alboleersin*

(*a*) *By heating with phenylhydrazine.* A mixture of luteoleersin (0·3 g.), anhydrous sodium acetate (0·3 g.), alcohol (12 ml.), water (3 ml.) and phenylhydrazine (0·2 g.) was heated under a reflux condenser for 1 hr. There was evolution of gas, presumably nitrogen. The mixture was cooled, diluted with water and carefully acidified with HCl. The buff-coloured solid which separated was recrystallized first from benzene and then from 70% aqueous methyl

alcohol. Yield, 70% of theoretical. Long colourless silky needles, M.P. 215–216° decomp., alone or mixed with authentic alboleersin. $[\alpha]^{18°}_{5461}+274°$, $[\alpha]^{18°}_{5780}+243°$ ($c=0{\cdot}5184$ in alcohol). (Found (Weiler): C, 67·35, 67·32; H, 8·34, 8·49%. Found (Schoeller): C, 67·36, 67·17; H, 8·57, 8·47%. $C_{26}H_{40}O_7$ requires C, 67·20; H, 8·68%.)

(*b*) *By catalytic reduction.* Luteoleersin (0·2 g.) dissolved in absolute alcohol (12 ml.) was reduced in an atmosphere of hydrogen with a Pd charcoal catalyst. The reduction was complete in 1–2 min. The alcoholic solution, now colourless, was filtered from the catalyst and alboleersin was precipitated by the addition of water. It was recrystallized from 70% aqueous methyl alcohol. Yield about 80%. Long colourless silky needles, M.P. 215° decomp. alone or mixed with authentic alboleersin.

*Conversion of alboleersin into luteoleersin*

Alboleersin (0·2 g.) dissolved in alcohol (4 ml.) was treated in the cold with 3 ml. of a solution containing anhydrous $FeCl_3$ (50 g.) in water (110 ml.). The solution, initially colourless, became momentarily dark green and finally deep golden in colour. After 5 min. it was diluted with water when luteoleersin separated as a yellow jelly which was filtered, washed, dried and recrystallized from light petroleum, B.P. 60–80°. Yield nearly quantitative. Yellow rods, M.P. 134–136°, alone or mixed with authentic luteoleersin. $[\alpha]^{18°}_{5461}+215°$, $[\alpha]^{18°}_{5780}+231°$ ($c=0{\cdot}4928$ g. in alcohol). (Found (Schoeller): C, 67·51, 67·54; H, 8·17, 8·25%. $C_{26}H_{38}O_7$ requires C, 67·50; H, 8·28%.)

Luteoleersin, in strongly acid (HCl) alcoholic solution, liberates iodine from potassium iodide. When carried out on a quantitative basis, this method, which gave theoretical results with benzoquinone, yielded with luteoleersin a figure which was about 10% too high. The reaction is complex, since the product which was isolated was not alboleersin, as would be expected, and further the same product was obtained by merely dissolving alboleersin in a mixture of alcohol and concentrated HCl. Luteoleersin (0·1021 g.) was dissolved in 95% alcohol (10 ml.). To the solution was added a mixture of 95% alcohol (20 ml.), 15% KI (10 ml.) and conc. HCl (10 ml.). The iodine liberated required 3·95 ml. *N*/10 thiosulphate, corresponding to a mol. wt. for luteoleersin of 512. ($C_{26}H_{38}O_7$ requires 462.) The solution from this and a number of similar titrations was diluted with water and the resultant precipitate was dried and crystallized from 65% aqueous methyl alcohol. Colourless masses of plates, M.P. 171–173°. The same product was obtained from alboleersin by dissolving it in 95% alcohol and adding concentrated HCl (using the same concentrations as for the titration but omitting KI). The resultant product, after recrystallization, melted at 174°, and had a mixed M.P. with the product from luteoleersin of 173–174°. (Found (Weiler), on the material from alboleersin: C, 67·22, 67·35; H, 8·11, 8·28%. $C_{26}H_{40}O_7$ requires C, 67·20; H, 8·68%.) The substance may be an isomeride of alboleersin. Its solutions readily oxidize in air, rapidly turning pink in colour. It is soluble in NaOH giving a deep yellow solution which rapidly turns red and a reddish orange flocculent substance separates. It is readily oxidized in alcoholic solution by $FeCl_3$ with the formation of a fine reddish colour. The product of the reaction, which is probably a quinone, is difficult to crystallize. It separates from aqueous methyl alcohol in long hair-like needles, reddish in colour, M.P. 129–131°. It gives a pale blue colour with concentrated $H_2SO_4$.

*Attempts to make derivatives of alboleersin and luteoleersin*

Gums or glasses, which were almost invariably extremely soluble in all organic solvents, and could not be crystallized, were given by alboleersin with the following reagents: (*a*) diazomethane; (*b*) dimethyl sulphate and alkali in acetone solution; (*c*) acetic anhydride, sodium acetate and glacial acetic acid; (*d*) 3:5-dinitrobenzoyl chloride and pyridine; (*e*) *p*-bromobenzoyl chloride and pyridine; and by luteoleersin with the following reagents: (*a*) diazomethane; (*b*) 2:4-dinitrophenylhydrazine; (*c*) semicarbazide hydrochloride.

SUMMARY

An account is given of the isolation in crystalline form, from the mycelium of *Helminthosporium leersii* Atkinson, grown on a Czapek-Dox glucose medium, of two hitherto undescribed mould metabolic products. These are luteoleersin, $C_{26}H_{38}O_7$, which was obtained in yellow blunt-ended needles, and alboleersin, $C_{26}H_{40}O_7$, which was obtained as colourless silky needles. Luteoleersin is believed to be a substituted quinone or semiquinone, and alboleersin the phenol corresponding to luteoleersin. The two substances are very readily interconvertible, luteoleersin giving alboleersin by simple reduction, and alboleersin giving luteoleersin by simple oxidation. The view is expressed that the two substances may form an oxidation-reduction system in the life processes of *H. leersii*.

We wish to express our indebtedness to Mr G. Smith and Mr W. K. Anslow for much assistance in the preparation of the mycelium of *H. leersii*, and the extraction from it of the crude metabolic products.

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# LVIII. THE DETERMINATION OF SMALL AMOUNTS OF DIMETHYLAMINE IN BIOLOGICAL FLUIDS

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(*Received 12 January 1938*)

LÖFFLER [1935] has stated that, of the volatile bases in human urine, ammonia and dimethylamine are present in greatest amount and that other methylamines are present only in traces. Attention has now been paid to dimethylamine in cow's urine; a suitable method for its determination in the presence of other nitrogenous bases is described in this paper.

Up to the present, the method of Weber & Wilson [1918] has been most successfully used for determining the distribution of volatile bases. In this method ammonia is absorbed with mercuric oxide, and methylamine is then determined by Van Slyke's amino-nitrogen method. Another portion of the solution is treated with nitrous acid which destroys ammonia and methylamine; dimethylamine is converted into the nitrosamine and trimethylamine is left unchanged. Steam distillation of the alkaline solution into standard acid enables the trimethylamine to be determined, while the nitrosamine which also distils over is reduced by Zn-HCl to a volatile base (stated by Weber & Wilson to be dimethylamine) which can be determined by difference after another distillation. The method is laborious and likely to yield misleading results if the many precautions given by the authors are not followed. The method is unsuitable for the quantities of amines usually found in biological material and is inaccurate in the presence of trimethylamine.

Katcher & Voroshilova [1934] have determined $NH(CH_3)_2$ in the presence of $NH_3$ by conversion into the $NH_4$ salt of dimethyldithiocarbamic acid (shaking the alkaline solution for 15 min. with carbon disulphide). The dithiocarbamate is then precipitated quantitatively as its insoluble copper salt by titration with 0·5*N* copper sulphate. This method is claimed to be accurate for quantities of about 0·25 g. of amine. Although $N(CH_3)_3$ forms an addition compound with $CS_2$, the colourless compound formed does not react with copper salts; $NH_2(CH_3)$ behaves in a similar manner to $NH(CH_3)_2$.

By modifying the dithiocarbamate technique, a simple sensitive method unaffected by the presence of $NH_2(CH_3)$ has been developed. The method does not distinguish between $NH(CH_3)_2$ and other secondary alkylamines; the latter, however, do not usually occur in biological material [Sidgwick, 1937]. The Cu salt of dimethyldithiocarbamic acid is insoluble in water but slightly soluble in organic solvents such as amyl alcohol, benzene and carbon tetrachloride. If a 5% solution of $CS_2$ in amyl alcohol is shaken with an alkaline aqueous solution containing $NH(CH_3)_2$ and a Cu salt, the upper layer rapidly assumes an amber tint. $NH_2(CH_3)$ forms a similar colour but of decreasing intensity as the alkalinity of the aqueous phase increases; this effect is more marked with $C_6H_6$ from

which the Cu salt of methyldithiocarbamic acid can be removed entirely by shaking with 0·1 *N* NaOH solution; the effect on the Cu dimethyldithiocarbamate is negligible under these conditions. There does not appear to be any decomposition of the monomethyl compound, which collects as a scum at the interface of the two liquids.

Before a solution of $CS_2$ in $C_6H_6$ will react with an aqueous solution of $NH(CH_3)_2$, a solvent mutually soluble in both phases must be added; amyl alcohol and ammonia are two such reagents. Since amyl alcohol reduces the effectiveness of separation of the copper salts, ammonia has been chosen for the method about to be described, with ammonium acetate as a source of buffering ammonium ions. A final shaking after the addition of acetic acid removes ammonia from the benzene layer, assists the clarification and increases the stability of the colour.

*Description and examination of the method*

*Solutions required:*

(A) A 5% solution of carbon disulphide in benzene.

(B) A solution of 20 g. ammonium acetate and 0·2 g. $CuSO_4$, $5H_2O$ in 30 ml. $H_2O$ is mixed in the cold with 10 g. NaOH in 25 ml. $H_2O$; to this are added 20 ml. $NH_4OH$ (sp. gr. 0·880) solution and the volume is made up to 100 ml.

(C) A 30% solution of acetic acid in water.

(D) A standard solution of $NH(CH_3)_2$ containing 10 mg. dimethylamine N per l.

To 10 ml. of a neutral aqueous solution containing 2–50 $\gamma$ dimethylamine nitrogen are added 1 ml. solution B and 10 ml. solution A. This mixture (in a large test tube) is warmed for 1 min. at 40°, stoppered and shaken vigorously for 30 sec. 1 ml. solution C is added and the mixture is shaken again for 30 sec., cooled, and centrifuged or allowed to stand until the upper layer is clear. The upper layer is decanted into a colorimeter cup and the depth of colour compared with that prepared in a similar way from a known amount of dimethylamine.

Some results given by this method when applied to solutions of dimethylamine hydrochloride are given in Table I.

Table I. *Determination of dimethylamine alone in solution*

| Standard contained 3·0 $\gamma$ N | | Standard contained 12·0 $\gamma$ N | | Standard contained 28·0 $\gamma$ N | |
|---|---|---|---|---|---|
| $\gamma$ N added | $\gamma$ N found | $\gamma$ N added | $\gamma$ N found | $\gamma$ N added | $\gamma$ N found |
| 2·0 | 1·94 | 8·0 | 7·84 | 20·0 | 18·5 |
| 3·0 | 3·12 | 10·0 | 9·92 | 24·0 | 23·9 |
| 4·0 | 4·02 | 13·0 | 12·72 | 29·0 | 29·1 |
| 6·0 | 6·16 | 17·0 | 16·80 | 34·0 | 33·2 |
| — | — | — | — | 40·0 | 39·8 |

It will be seen that the interaction of amine with carbon disulphide and copper has proceeded to completion, and that the depth of colour obeys Beer's law. If the dimethylamine nitrogen exceeds 50 $\gamma$ this agreement ceases owing to the supersaturation of the benzene with copper salt and its subsequent precipitation.

Before it could be decided whether this method was affected by the presence of mono- and tri-methylamines, it was necessary to free solutions of these bases

from dimethylamine. The 33% aqueous solutions which were used for these experiments were found to contain only a trace (0·05%) of dimethylamine in the case of the primary amine, but over 1·5% in the case of the tertiary amine. Purification of the former was readily accomplished by recrystallization of its hydrochloride from alcohol, but this method could not be applied to trimethylamine. This base was purified by treatment with nitrous acid, alkaline distillation into hydrochloric acid, evaporation of the distillate to dryness and recrystallization from alcohol-ether. All traces of dimethylnitrosamine had to be removed since its aqueous solution appeared to be decomposed by sunlight to dimethylamine. The purified amine solutions were found to be free from formaldehyde and to give no reaction with Schryver's reagent. This is in agreement with the finding of Reay [1936, 1] that the positive reaction of these amines with this reagent previously observed by him [1936, 2] and by Tankard & Bagnall [1926], was due to the use of impure solutions and not to any fault in the Schryver technique.

Table II gives the results for the above method with dimethylamine in the presence of larger concentrations of the other methylamines and of ammonia. Each result is calculated from the mean of 4 colorimeter readings. The $NH_3$, $NH_2(CH_3)$ and $N(CH_3)_3$ were added as solutions containing 100$\gamma$ N per ml., and the mixed solutions were adjusted by dilution or concentration to 10 ml. in all cases.

Table II. *Determination of dimethylamine in a mixture of amines*

| Bases added ($\gamma$ N) | | | | Dimethylamine found |
|---|---|---|---|---|
| $NHMe_2$ $\gamma$ N | $NH_3$ | $NH_2Me$ | $NMe_3$ | $\gamma$ N |
| 2·0 | 1000 | — | — | 1·98 |
| | — | 1000 | — | 2·08 |
| | — | — | 1000 | 2·01 |
| | 10 | 10 | 10 | 2·01 |
| | 100 | 100 | 100 | 2·14 |
| | 500 | 500 | 500 | 2·47 |
| 10·0 | 500 | — | — | 9·95 |
| | — | — | 500 | 9·76 |
| | 10 | 10 | 10 | 9·95 |
| | 100 | 100 | 100 | 9·95 |
| | 500 | 500 | 500 | 10·10 |
| 50·0 | 10 | 10 | 10 | 50·05 |
| | 100 | 100 | 100 | 50·10 |
| | 500 | 500 | 500 | 50·25 |

Table II shows that in mixtures containing large amounts of amines other than $NH(CH_3)_2$ slightly higher values than theoretical for that base are given. A mixture containing in 10 ml. 2·5 mg. N as $N(CH_3)_3$ and 0·1 mg. N as $NH_2(CH_3)$ only, gave a coloration equivalent to 0·83$\gamma$ N as dimethylamine, although the separate constituents gave no coloration. It seems that the molecular rearrangement $NH_2Me + NMe_3 = 2NHMe_2$ occurs probably only during the shaking with carbon disulphide and copper solution when the dimethylamine is removed as soon as formed.

*Application of the method to urine*

To establish as far as possible that no normal urinary constituents interfere with this determination some samples of urine were examined before and after the addition of known amounts of dimethylamine. The method consisted of

distilling 5 ml. with 50 ml. $H_2O$ and 10 ml. *N* NaOH until 40 ml. of distillate had been collected. This was made up to 50 ml. and 5 ml. were taken for the determination of dimethylamine. At the same time a direct determination, omitting the distillation, was made on 5 ml. of urine diluted 10 times. The results (Table III) showed in the case of cow's urine that little advantage was gained by distillation.

Table III. *Dimethylamine in urine* (*mg. N/litre*)

| Source of sample | Method | Original $NHMe_2$ content | $NHMe_2$ added | Total $NHMe_2$ found | Added $NHMe_2$ found |
|---|---|---|---|---|---|
| Cow A | Direct | 27·6 | 5·0 | 32·2 | 4·6 |
| | Distilled | 28·1 | 5·0 | 33·3 | 5·2 |
| Cow B | Direct | 40·4 | 10·0 | 50·3 | 9·9 |
| | Distilled | 40·4 | 10·0 | 50·0 | 9·6 |
| Cow C | Direct | 18·8 | 6·0 | 25·3 | 6·5 |
| | Distilled | 19·4 | 6·0 | 25·8 | 6·4 |
| Human | Direct | 5·8 | 10·0 | 14·5 | 8·7 |
| | Distilled | 7·6 | 10·0 | 17·2 | 9·6 |

*Reduction of the nitrosamine in the Weber & Wilson method*

It was found that the nitrosamine obtained by the action of nitrous acid on dimethylamine was not reduced to the parent amine by Zn-HCl, as stated by Weber & Wilson, but mainly to unsymmetrical dimethylhydrazine ($N(CH_3)_2.NH_2$) which is strongly dissociated, and can be determined by steam distillation from an alkaline solution. Of many methods examined that of Jones & Kenner [1932] was alone found applicable to the reduction of the nitrosamine to the original amine. If 2 ml. of a 20 % solution of $Cu_2Cl_2$ in HCl and 2 ml. conc. HCl are added to a solution of the nitrosamine in 5 ml. water, the nitrosamine is converted quantitatively into $NH(CH_3)_2$ when the mixture is slowly raised to the boiling point, and the base can then be isolated by distillation from alkaline solution. (The distillation should be done in an all-glass apparatus as dithiocarbamates and derivatives of $NH(CH_3)_2$ are used as rubber "accelerators".) In the presence of $N(CH_3)_3$ more than the theoretical amount of $NH(CH_3)_2$ could be recovered owing to the oxidation of the tertiary amine to the nitrosamine. On standing at room temperature about 1 %, and at 70° 13 %, of the tertiary amine was thus changed. Wegler & Frank [1936] have observed the same effects on bornyl dimethylamine and some aromatic amines and they state that the reaction is slowest at low temperatures and in the presence of strong mineral acids. Taylor [1928; 1929] has shown that the activity of nitrous acid towards primary and secondary alkylamines is decreased in the presence of mineral acids and it is probable that the conditions stipulated in the Weber & Wilson method are the most suitable.

SUMMARY

A colorimetric method for the determination of small amounts of dimethylamine, in the presence of other volatile bases, as copper dimethyldithiocarbamate in solution in organic solvents is described. The interference of methylamine is avoided by making the aqueous phase alkaline.

The oxidation of small amounts of trimethylamine to dimethylnitrosamine by nitrous acid is reported.

The author is indebted to Prof. H. D. Kay for his kindly interest in this work.

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# LIX. IDENTIFICATION OF THE PROTOPORPHYRIN IN SHEEP'S LIVER

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THAT very small quantities of protoporphyrin occur normally in ox liver has been demonstrated spectroscopically by Fischer *et al.* [1925]. Since the melting point of the ester was not recorded it is impossible to say whether this pigment belonged to the I or III series of compounds, derivable from aetioporphyrins I and III respectively, although up to the present no protoporphyrin of series I has been found to occur in nature.

Van den Bergh *et al.* [1932] demonstrated by a perfusion technique that the liver was capable of converting natural protoporphyrin added to the blood stream into coproporphyrin, which was excreted in the bile. Here again, the coproporphyrin found was only identified spectroscopically. However, since a complete disintegration and re-arrangement of the molecule would be necessary to convert a series III into a series I derivative, it may be assumed that in the experiments of the Dutch workers their protoporphyrin passed smoothly into the corresponding coproporphyrin III. The same workers proved that the blood of normal healthy persons contained traces of a protoporphyrin [cf. Watson, 1937] which was isolated and identified as protoporphyrin of series III by Grotepass [1937].

A small quantity of coproporphyrin is normally present in the urine of healthy individuals and this has recently been shown to be coproporphyrin I [Fink & Hoerburger, 1934]. Its configuration precludes its origin from normal haemoglobin, which is a series III derivative; rather is it suggested that it may be a by-product in the synthesis of the blood pigment, and a scheme has been put forward to illustrate such a mechanism [Rimington, 1936]. The possibility remained, however, that it might have arisen from a series I protoporphyrin by transformation in the liver as in the Van den Bergh experiments. The protoporphyrin of sheep's liver has therefore been isolated and converted into the corresponding mesoporphyrin, and this has been shown to be identical with mesoporphyrin IX, thereby establishing it and the parent protoporphyrin as series III derivatives. The coproporphyrin I of the urine must consequently have some independent origin.

## EXPERIMENTAL

Experience had shown that sheep's liver was a richer source of protoporphyrin than was ox liver. Five sheep's livers (wt. 2660 g.) were minced within half an hour of the death of the animals and the pulpy mass was stirred with 2 l. glacial acetic acid. To the mixture were added 3 l. ether whilst stirring vigorously; after 2 hr. the extract was squeezed off through muslin and the residue was re-extracted with a further 2 l. ether. The combined ether extracts were washed repeatedly with water in a large separating funnel, and the porphyrin was extracted by repeated shakings with 10% HCl. To the resulting blue-green solution enough saturated potassium acetate was added to make it neutral to

Congo red and the pigment was taken up by shaking with ether. The protoporphyrin spectrum was clearly visible in this red solution, but shaking with 1% HCl removed a small quantity of a coproporphyrin (chloroform-insoluble, spectrum 625m$\mu$. etc.).

After evaporation of the remaining ethereal solution, the solid residue was taken up in about 20 ml. of a mixture of acetic acid and HI (7·5 : 1) and the solution was maintained at boiling temperature for 3 min. After cooling and addition of ether, the excess of iodine and acid was removed by repeated shaking with a mixture of sodium acetate and sodium sulphite. The ether phase showed the absorption spectrum of mesoporphyrin. After evaporation to dryness the pigment was esterified at room temperature in saturated methyl alcoholic HCl and the ester was transferred to chloroform. The washed chloroform layer was then diluted with several volumes of ether and the pigment shaken out into 5% HCl. From this it was driven back to a small volume of ether by means of potassium acetate; the ethereal liquid was washed, dried and allowed to evaporate to dryness. The crystalline residue consisted of clusters of small needle-like prisms. It was washed with light petroleum, ether and methyl alcohol and then recrystallized by dissolving in the minimum quantity of chloroform, adding about 5 vols. dry ether and an equal quantity of petroleum and allowing the mixture to remain overnight at 0°.

The product melted at 207–210° and, after a further recrystallization, at 211–213°. Mixed with mesoporphyrin IX dimethyl ester (M.P. 215–216°, kindly supplied by Prof. H. Fischer) it had M.P. 211–215°. The original protoporphyrin was thereby identified as possessing a similar constitution to blood pigment derived from aetioporphyrin III.

## Summary

The protoporphyrin normally present in sheep's liver has been isolated and converted into a mesoporphyrin (M.P. 211–213°) identical with mesoporphyrin IX. It is therefore a series III derivative and cannot be the source of the coproporphyrin I found in traces in normal urine.

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# LX. A NOTE ON THE DIGESTION OF FATS BY PANCREATIC LIPASE

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(*Received 19 January 1938*)

Fats have for some time been recognized as basal dietary constituents, though even now it is not definitely established whether or not these constituents should be regarded as essential food factors. That animals eat a variety of fats, both of animal and vegetable origin, is widely recognized and there is evidence that man can digest a number of fats other than those which are normally included in his diet.

Special consideration was given to this type of food when a Committee of the Royal Society [1918–19] considered the possibility of utilizing fatty acids as food. Burns & Sharpe [1918–19] concluded that the fatty acids of whale oil were as thoroughly digested and absorbed as was the hardened oil from which they were prepared. Gardner & Fox [1919] found that cocoabutter could be satisfactorily utilized by man. Barbour [1934], working with rats, showed that peanut oil or cottonseed oil could be effectively metabolized when it constituted 20% of the diet. Langworthy & Holmes [1917, 1], from experiments on man, found that olive oil, cottonseed oil, peanut oil, coconut oil, sesame oil and cocoabutter could safely be included in his diet, but concluded that the limit of tolerance was less for cocoabutter than for the other fats studied. With animal fats these observers [1915] found the coefficient of digestibility of butter to be 97·1%, of lard 97·3%, of beef fat 93·1% and of mutton fat slightly less, 87·6%; for cream the figure was 96·9% [1917, 2].

In the experiments just quoted the subjects were left entirely free as to the amount of food they consumed and wide variations were noted. For instance, with beef fat the average amount taken per day was 100 g. and with mutton fat only 53 g. This work demonstrated that various fats could be adequately digested and assimilated by man, but afforded no information as to the respective rates of digestion of the fats. Weber & King [1935] found that the $\alpha$- and $\beta$-monoglycerides of lauric, myristic, palmitic and stearic acids were hydrolysed with equal facility by pancreatic lipase, but on the whole there appears to be little experimental evidence as to the comparative rates of digestion of different fats.

## Experimental

Various methods for emulsifying fats have been used by different workers. A method in general use involves the use of alcohol, but other methods have been suggested such as the use of gum acacia or neutral sodium oleate as emulsifying agents [Weinskin & Wynne, 1935–36]. Such methods have no direct application to digestion in man and it was desirable to use the fat in a form in which it could be taken as food. At first an emulsion in water [Plimmer, 1933] was tried, but this method could not be used because it was applicable only to some and not to all fats. Reconstituted creams in which various fats were substituted for butter were next tried and this method proved satisfactory.

*Preparation of creams*

The creams used in these experiments were made with a well-known type of machine and it was found that once the machine was "run in" a standard product could always be obtained. It had been shown previously [Hartwell, 1937] that the best emulsification was obtained with the proportion of 57 g. butter to 105 ml. milk; the cream so made contains approximately 33 % of fat. With other fats which contained little or no water, 47 g. fat were added to 105 ml. milk and 10 ml. water. When creams were kept overnight they were placed in a refrigerator, previous experiments [Hartwell, 1937] having shown that keeping them in this way made no difference to their rate of digestion. Such creams were allowed to stand for half an hour after being made, so that the layer of air bubbles which came to the top could be removed. If this precaution were not taken, a thick crust formed which rendered an even mixing of the cream impossible.

Beef, mutton and bacon fats were prepared in the following way. Any lean was removed and the fat was cut into small pieces and heated in a gas oven for $\frac{1}{2}$–$\frac{3}{4}$ hr., after which the liquid fat was poured off, allowed to cool and used for the experiments.

In making the creams the fat was melted and the milk was heated to boiling point; the machine was also heated.

Butter cream was made by pumping the mixture once through the machine. With all the other fats the mixture was pumped through three times to ensure complete emulsification, because it was found that some fats (especially olive oil and lard) were apt to oil out on top if only put through once. Some preliminary experiments were made with creams which had been put through the machine six and nine times. These creams, however, digested at the same rate as those which had been put through only three times, therefore it seemed unnecessary to take this extra precaution. Microscopic examination showed that all the creams consisted of very small fat globules, and no differences in the sizes of the fat droplets could be detected.

*Method of experiment*

The commercial form of pancreatic substance used as a source of lipase was that found by Clifford [1934] to be suitable for this type of work. In a typical experiment 50 ml. cream, 175 ml. distilled water and 25 ml. of 2–4 % pancreatic substance (the exact strength depending on the activity of the sample) were used.

The cream was measured in a 50 ml. graduated cylinder, and washed into a flask with the distilled water, the pancreatic substance being added last. The reagents were mixed cold, not warmed separately as is usual in digestion experiments.

The sample of lipase used was active at room temperature (19–19·5°), and in some cases digestion started so rapidly that the initial stages would not have been recorded had the reagents been at 38° when mixed.

Immediately after mixing 20 ml. were taken for titration, further 20 ml. samples being removed every 15 min. To the 20 ml. digestion mixture were added approximately 150 ml. boiling distilled water and 2 ml. phenolphthalein and the whole was titrated hot with 0·1 *N* NaOH. The flasks were placed in a large water bath and the temperature was maintained at 38–39°.

Butter cream was used as a standard for comparison and control experiments were made with milk and with boiled pancreas substance and cream. The fats

used were almond oil, arachis oil, bacon fat, beef fat, beef oleo, beef stearin, castor oil, cocoabutter, coconut oil, cod liver oil, cottonseed oil, lard, mutton fat, olive oil, palm oil, palm kernel oil, premier jus, soya bean oil, hardened whale oil and hardened arachis oil.

Blank titrations of the creams alone gave 0·25–0·3 ml. of 0·1*N* NaOH for butter. The other creams ranged from 0·2 to 0·3 ml. except beef oleo, premier jus and castor oil which gave slightly higher values, 0·4–0·45 ml. The slightly higher figure for the first titrations of some creams is due to the rapid start of digestion. Palm oil had an appreciably higher value, 1·8 ml., and owing to its yellow colour, it was difficult to judge the end point of the titration.

## Results and comments

Butter cream was taken as a standard for comparison and each experiment included three or four of the other creams. These were arranged in different combinations and at least three experiments were made with each.

A comparison of lard, fat from smoked bacon and fat from green bacon showed that there was no difference in the rates of digestion of these three fats. Fat prepared from beef suet and from scrap beef fat also gave similar digestion curves, as did mutton suet fat and scrap mutton fat, so it was concluded that it was unnecessary to use more than one type of fat from any animal.

When it was found that beef and mutton fats were digested so slowly when compared with butter fat, it was thought possible that heating the fats might have had some detrimental effect. In order to see if this supposition were true two samples of butter were heated in a gas oven, one sample at the same time as some beef suet, and the other sample with some mutton suet. The digestion of the fat in creams made from these heated butters showed no alteration in rate, therefore it was unlikely that heating the beef and mutton fats was the cause of the slower digestion.

The majority of the fats used were digested by lipase much more slowly than was butter fat, three only showing a more rapid rate of digestion. Coconut oil was digested more rapidly than any other fat; three separate samples were used and all gave similar digestion curves. Palm kernel oil also showed a rapid rate of digestion, but not so rapid as the coconut oil. Castor oil was digested more rapidly than butter, but the curve obtained with this fat was of rather a different type from the other curves.

Almond oil, arachis oil, bacon fat, beef fat, beef oleo, beef stearin, cocoabutter, cod liver oil, cottonseed oil, lard, mutton fat, olive oil, premier jus, soya bean oil, hardened whale oil and hardened arachis oil were digested at a much slower rate than butter and all at approximately the same rate. This is in agreement with the experiments of Gardner & Fox [1919] who found that cocoabutter was rather less digestible than butter. Palm oil digested slowly, but could hardly be compared with the others, owing to the difficulty in judging the end point because of the orange colour of the fat itself. The experiments, however, served to demonstrate that palm kernel oil belongs to the group of fats which are digested slowly by pancreatic lipase.

That coconut oil is digested so much more quickly than butter may be of dietetic interest in that this oil is said to be used for the making of cream buns. It seems possible that if margarines contain this fat they may be more rapidly digested than butter. Except in the case of castor oil, the rate of digestion runs parallel with the saponification value of the fats. Thus of the fats used, coconut oil has the highest saponification value [Bolton, 1928] and is the most rapidly

digested. According to Lewkowitsch [1923] palm kernel oil is very nearly related in its chemical composition to coconut oil and its digestion curve (Fig. 1) is nearer to that of coconut oil than that of any other fat. Wright & Mitchell [1921] state that in butter and coconut oil fatty acids of low molecular weight

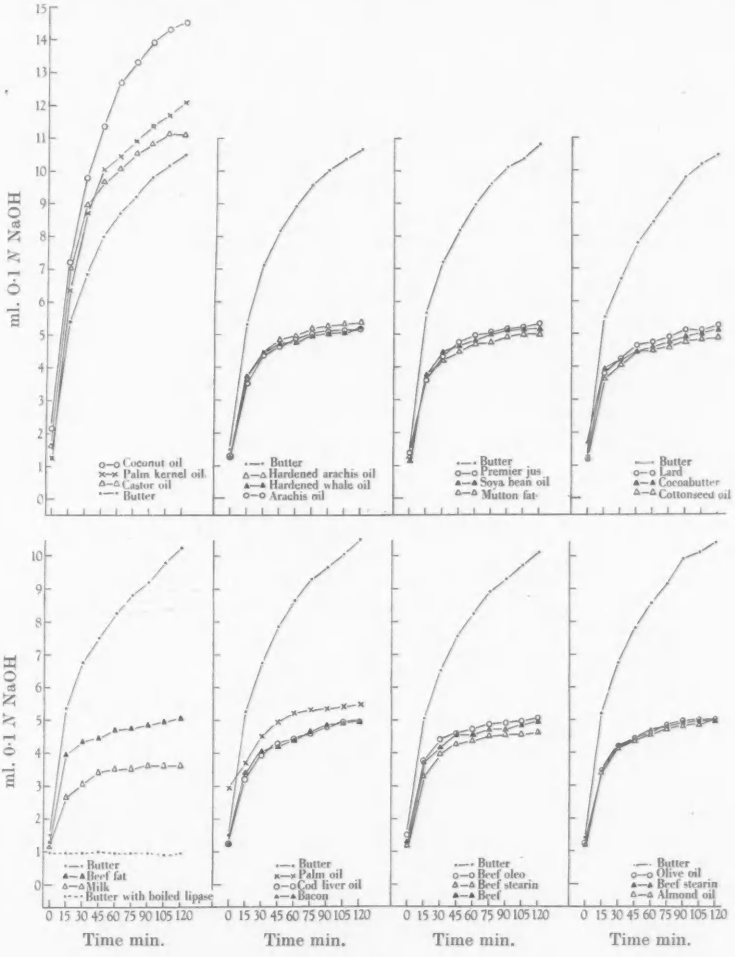


Fig. 1.

are present "to some notable extent", while as a general rule natural oils and fats rarely yield fatty acids of that description. It is possible that this may be correlated with the more rapid rate of digestion of coconut oil and butter fat by lipase. Evans *et al.* [1934] put coconut oil first in order of fats able to spare vitamin B. If, as suggested by Burr *et al.* [1932] linoleic is an essential fatty

acid, then coconut oil which contains 1% [Chemists' Year Book 1934] and is rapidly digested, may prove a more valuable food than has hitherto been supposed.

SUMMARY

1. Coconut oil was digested by pancreatic lipase more rapidly than any other fat.

2. Palm kernel oil and castor oil were digested more rapidly than butter fat, while almond oil, arachis oil, bacon fat, beef fat, beef oleo, beef stearin, cocoabutter, cod liver oil, cottonseed oil, lard, mutton fat, olive oil, palm oil, premier jus, soya bean oil, hardened whale oil and hardened arachis oil were digested appreciably more slowly, and at approximately equal rates.

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# LXI. VITAMIN C STUDIES IN THE RAT. THE EFFECT OF SELENIUM DIOXIDE, SODIUM SELENATE AND TELLURATE

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(*Received 25 November 1937*)

Selenium poisoning in rats, either from sodium selenite [Franke & Potter, 1935] or from certain natural plant intoxicants [Franke, 1934], results in extensive pathological changes in the liver. The severity of the pathological changes in other organs depends on the extent to which the liver cells are regenerated as the result of necrosis.

Reduction of the toxic selenite and tellurite to the insoluble and relatively non-toxic elemental form by living cells has been reported from many laboratories. Respiration studies show that the $O_2$ uptake of yeast cells [Potter & Elvehjem, 1936] and the rate of $CO_2$ evolution during yeast fermentation [Moxon & Franke, 1935] are inhibited by sodium selenite. The reaction between the selenite and glutathione to give oxidized glutathione and selenium [Bersin, 1935] has been suggested by Potter & Elvehjem [1936] as an explanation for the toxic action of selenite in glycolysis.

The respiratory mechanism of animal tissues is generally considered to be much more complex than that in yeast. The role of vitamin C in tissue respiration is not completely understood, but glutathione has been regarded as an essential factor in the stabilization of this vitamin in tissue extracts [De Caro & Giani, 1934].

The present investigation was undertaken to study the effect of certain salts causing selenium and tellurium poisoning in rats on the vitamin C content of organs and to determine whether glutathione exerts a stabilizing influence on the reduction of these salts by the vitamin *in vitro*.

## Experimental

Young albino rats of 90 g. wt., previously fed exclusively on the complete stock ration sold as Ralston Purina Dog Chow, were placed on basal diets devoid of vitamin C consisting of:

(*a*) 6% casein, 45% cornstarch, 15% sucrose, 4% Osborne and Mendel salts, 5% cod liver oil, 20% lard and 5% dried yeast.

(*b*) 18% casein, 32% starch, 15% sucrose, 4% salts, 5% cod liver oil, 15% lard, 9% dried yeast and 2% agar-agar.

(*c*) 52% casein, 3% salts, 30% butterfat, 15% lard, with the addition of 0·4 g. dried yeast daily to each rat. This diet is referred to in Tables I and II as "carbohydrate-deficient".

Fresh food (10 g. per rat) and water *ad lib.* were given daily. Adequate growth response was shown to occur with these diets in a 10-day preliminary test period.

The addition of selenium dioxide at a level of 22–35 p.p.m. of Se resulted in the refusal of the rats to eat the diets. The basal diets were then given for 3 days and the increased body weight was taken as the initial body weight. The average age of the rats at this stage of the experiment was 7–8 weeks.

Subcutaneous injections of the salts at levels determined by their respective toxicity were then given. At the end of the brief experimental period, the rats were killed by stunning, the organs were immediately removed (unless otherwise noted), weighed, extracted with 4% trichloroacetic acid and analysed in the usual manner for ascorbic acid by rapid titration with 2:6-dibromophenolindophenol blue.

*Experiments* in vivo

In these tests the rats injected with selenium and tellurium salts showed at necropsy the general symptoms—garlic odour of the liver and autolysis of the gut—mentioned in detail by Sollmann [1936]. In such a brief experimental period the marked necrosis of the liver noted in the prolonged feeding of plant intoxicants by Franke [1934] was not observed, but definite and rapid increases in weight of this organ were recorded.

The data in Tables I and II indicate that the percentage decrease of ascorbic acid in the organs, in accordance with previously recorded work on growth response by Schneider [1936], was not influenced by the sulphydryl composition of the diet but depended on the amount of toxic salt injected and on the duration of the test period. The concentration of the vitamin, in contrast to the results of giving appreciably greater quantities of metallic salts such as copper sulphate, lead acetate, arsenic pentoxide, mercuric chloride, cadmium sulphate and manganese chloride, which had no apparent effect [Svirbely, 1936], was generally decreased in all the organs. A possible explanation for this decrease will be discussed in the next section.

A positive silver nitrate test for ascorbic acid has been reported by Mouriquand & Couer [1935] to persist in the adrenals of normal guinea-pigs for as long as 4 days after the death of the animal. Tissue extracts of normal rats which had been killed by stunning and whose intact carcasses had been allowed to stand at room temperature or in the ice-box for a period of 14 hr. showed, in comparison with extracts from rats fasting a like interval but killed just prior to extraction of the organs, an appreciable decrease in the vitamin C content of the organs. This decrease is more marked per g. tissue if correction for the post-mortem autolysis of the liver and gut be taken into consideration. The time factor is not the sole explanation for the decrease in vitamin C concentration in the rats injected with lethal doses of the salts (Table I *), since the control rats in the same series were killed by stunning and allowed to stand at room temperature for 4 hr. before extraction of the organs was carried out. However, in order to avoid errors in the interpretation of data, the estimation of vitamin C should be made as soon as possible after death of the animals unless the carcasses are frozen thoroughly.

*Estimation of vitamin C* in vitro

Preliminary experiments indicated that reduction of selenium dioxide or sodium selenate to the colloidal state occurred more slowly with glutathione or cysteine (as the hydrochloride) than with ascorbic acid. Reduction of sodium tellurate in concentrations possessing the same degree of toxicity as the selenium salts was brought about more rapidly by cysteine than by glutathione, but did not occur with ascorbic acid. The rapid reduction of tellurate by cysteine, even in the presence of vitamin C, may account for the pigmented urine and dark

Table I. *Effect of subcutaneous injections of selenium dioxide, sodium selenate and tellurate in 7–8-week-old rats. Rats fasted during test period*

| Rats had been fed dog chow | No. and sex of animals | Av. initial wt. g. | Av. final wt. g. | Amnt. salt injected mg. | Test period hr. | Vitamin C content of organs: Adrenals mg. per g. | Liver mg. per g. | Gut mg. per g. | Spleen mg. per g. | Adrenals mg. per organ | Liver mg. per organ | Gut mg. per organ | Spleen mg. per organ | Total amnt. in organs extracted | % decrease |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Selenium dioxide | 3♂* | 128 | 115 | 0·5 | 15 | 1·0 | 0·08 | 0·19 | 0·17 | 0·02 | 0·53 | 0·75 | 0·05 | 1·35 | 22 |
| Selenium dioxide | 3♂* | 121 | 105 | 0·5 | 15 | 0·5 | 0·08 | 0·22 | 0·17 | 0·01 | 0·54 | 0·81 | 0·06 | 1·42 | 18 |
| Sod. selenate | 3♂* | 126 | 117 | 0·85 | 15 | 0·5 | 0·08 | 0·18 | 0·15 | 0·01 | 0·51 | 0·69 | 0·05 | 1·26 | 28 |
| Sod. tellurate | 3♂* | 120 | 112 | 10·0 | 15 | 0·5 | 0·08 | 0·15 | 0·05 | 0·01 | 0·41 | 0·53 | 0·02 | 0·97 | 44 |
| Sod. tellurate | 3♀* | 128 | 118 | 10·0 | 14 | 0·4 | 0·09 | 0·12 | — | 0·01 | 0·46 | 0·38 | — | 0·85 | 45 |
| Controls | 3♂* | 120 | 103 | — | 19 | 1·25 | 0·17 | 0·21 | 0·27 | 0·03 | 0·78 | 0·84 | 0·07 | 1·74 | — |
| Controls † | 3♀ | — | 115 | — | 14 | 1·27 | 0·12 | 0·18 | 0·13 | 0·04 | 0·56 | 0·62 | 0·07 | 1·29 | 23 |
| Controls ‡ | 3♀ | — | 118 | — | 14 | 0·78 | 0·11 | 0·24 | 0·17 | 0·02 | 0·59 | 0·77 | 0·06 | 1·44 | 11 |
| Controls § | 3♀ | — | 108 | — | 14 | 2·06 | 0·17 | 0·20 | 0·24 | 0·06 | 0·71 | 0·80 | 0·11 | 1·68 | — |
| Selenium dioxide | 5♀ | 120 | 107 | 0·3 | 16 | 1·11 | 0·10 | 0·20 | 0·19 | 0·03 | 0·51 | 0·75 | 0·06 | 1·35 | 17 |
| Sod. selenate | 3♀ | 119 | 102 | 0·5 | 16 | 1·00 | 0·07 | 0·16 | 0·18 | 0·03 | 0·35 | 0·70 | 0·05 | 1·13 | 30 |
| Sod. tellurate | 3♀ | 118 | 104 | 5·0 | 17 | 1·52 | 0·14 | 0·17 | 0·11 | 0·04 | 0·64 | 0·64 | 0·06 | 1·38 | 15 |
| Controls | 4♀ | 124 | 109 | — | 17 | 2·26 | 0·17 | 0·21 | 0·14 | 0·06 | 0·64 | 0·86 | 0·07 | 1·63 | — |

* Salts injected were in mg. per 100 g. initial body wt. All rats died on this dosage. The organs were removed and extracted about 4 hr. after death of the animals.

† Room temperature 26°, ‡ Ice-box } Organs removed for extraction 14 hr. after death of animals.

§ Fasting control rats

Table II. *Effect of selenium dioxide on rats fed on various diets. Rats fasted during test period. Av. age of rats in these tests was 59–62 days*

| Diet and injections | No. and sex of animals | Av. initial wt. g. | Av. final wt. g. | Amnt. salt injected mg. | Test period hr. | Vitamin C content of organs | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Adrenals | Liver | Gut | Spleen | Adrenals | Liver | Gut | Spleen | Total amnt. in organs extracted | % decrease |
| | | | | | | mg. per g. | | | | mg. per organ | | | | | |
| Repeated injections of $SeO_2$* | | | | | | | | | | | | | | | |
| 6% casein | 3♀ | 117 | 105 | 0·8 | 43 | 1·58 | 0·06 | 0·23 | 0·12 | 0·05 | 0·35 | 0·67 | 0·05 | 1·12 | 35 |
| 6% casein | 3♀ | 127 | 117 | — | 40 | 1·50 | 0·13 | 0·24 | 0·13 | 0·03 | 0·60 | 1·02 | 0·07 | 1·72 | — |
| 18% casein | 3♀ | 152 | 134 | 1·8 | 48 | 1·50 | 0·07 | 0·22 | 0·14 | 0·06 | 0·47 | 0·79 | 0·07 | 1·39 | 16 |
| 18% casein | 3♀ | 150 | 133 | — | 48 | 3·32 | 0·17 | 0·16 | 0·23 | 0·12 | 0·77 | 0·63 | 0·15 | 1·67 | — |
| Carbohydrate-deficient | 4♂ | 167 | 143 | 2·8 | 48 | 2·03 | 0·11 | 0·27 | 0·11 | 0·06 | 0·89 | 1·09 | 0·09 | 2·13 | 35 |
| | 3♂ | 188 | 164 | — | 48 | 3·57 | 0·28 | 0·28 | 0·09 | 0·10 | 1·69 | 1·42 | 0·08 | 3·29 | — |
| Dog chow | 5♂ | 154 | 128 | 2·8 | 48 | 1·56 | 0·07 | 0·26 | 0·09 | 0·05 | 0·61 | 1·19 | 0·05 | 1·90 | 24 |
| Dog chow | 4♂ | 155 | 122 | — | 48 | 3·00 | 0·20 | 0·28 | 0·16 | 0·07 | 1·03 | 1·30 | 0·10 | 2·50 | — |
| Single injection of $SeO_2$ | | | | | | | | | | | | | | | |
| Dog chow | 4♂ | 149 | 149 | 2·0 | 4 | 1·16 | 0·15 | 0·36 | 0·14 | 0·03 | 1·08 | 1·92 | 0·09 | 3·12 | 21 |
| Dog chow | 2♂ | 151 | 151 | — | 4 | 2·05 | 0·26 | 0·32 | 0·13 | 0·04 | 1·79 | 1·95 | 0·17 | 3·95 | — |
| Carbohydrate-deficient | 3♂ | 158 | 149 | 1·0 | 20 | 2·44 | 0·10 | 0·32 | 0·20 | 0·07 | 0·82 | 1·58 | 0·11 | 2·57 | 22 |
| | 2♂ | 191 | 181 | — | 20 | 3·18 | 0·22 | 0·23 | 0·19 | 0·10 | 1·68 | 1·36 | 0·18 | 3·32 | — |

* Subcutaneous injections: 0·3 mg. on first day at noon; 0·5 mg. 28 hr. after 1st injection; 1–2 mg. 45 hr. after 1st injection.

Table III. *Estimation of vitamin C in the presence of selenium dioxide, sodium selenate and tellurate*

| Ascorbic acid added mg. | Glutathione mg. | Selenium dioxide mg. | Ascorbic acid estimated mg. | % decrease | Remarks | Ascorbic acid added mg. | Glutathione mg. | Na selenate mg. | Ascorbic acid estimated mg. | % decrease | Remarks | Ascorbic acid added mg. | Glutathione mg. | Na tellurate mg. | Ascorbic acid estimated mg. | % decrease |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Titrated within 2–5 min. | | | | | | | | | | | | | | | | |
| 0·60 | — | — | 0·60 | — | — | — | — | — | | | — | — | — | — | — | — |
| 0·60 | — | 0·1 | 0·58 | 3 | — | 0·60 | — | 0·1 | | | — | 0·60 | — | 1·0 | 0·59 | 1 |
| 0·60 | — | 0·3 | 0·56 | 6 | — | 0·60 | — | 0·3 | No appreciable decrease | Less than 1·0 | — | 0·60 | — | 3·0 | 0·59 | 1 |
| 0·60 | — | 0·5 | 0·50 | 8 | — | 0·60 | — | 0·5 | | | — | 0·60 | — | 5·0 | 0·59 | 1 |
| 0·60 | — | 1·0 | 0·46 | 23 | — | 0·60 | 2·0 | 0·1 | | | — | 0·60 | — | 10·0 | 0·58 | 3 |
| 0·60 | 2·0 | 0·1 | 0·60 | — | — | 0·60 | 2·0 | 0·3 | | | — | 0·60 | 2·0 | 1·0 | 0·60 | — |
| 0·60 | 2·0 | 0·3 | 0·59 | 1 | — | 0·60 | 2·0 | 0·5 | | | — | 0·60 | 2·0 | 3·0 | 0·60 | — |
| 0·60 | 2·0 | 0·5 | 0·57 | 5 | — | — | — | — | | | — | 0·60 | 2·0 | 5·0 | 0·60 | — |
| 0·60 | 2·0 | 1·0 | 0·52 | 13 | — | — | — | — | | | — | 0·60 | 2·0 | 10·0 | 0·60 | — |
| Titrated after standing 20 min. | | | | | | | | | | | | | | | | |
| 0·485 | — | 0·30 | 0·42 | 11 | * | — | — | — | — | — | — | — | — | — | — | — |
| 0·485 | — | 0·50 | 0·38 | 21 | * | — | — | — | — | — | — | — | — | — | — | — |
| 0·485 | — | 1·00 | 0·30 | 38 | * | — | — | — | — | — | — | — | — | — | — | — |
| 0·485 | 2·0 | 0·30 | 0·45 | 6 | — | — | — | — | — | — | — | — | — | — | — | — |
| 0·485 | 2·0 | 0·50 | 0·42 | 13 | — | — | — | — | — | — | — | — | — | — | — | — |
| 0·485 | 2·0 | 1·00 | 0·38 | 21 | — | — | — | — | — | — | — | — | — | — | — | — |
| Titrated after standing 2 hr. | | | | | | | | | | | | | | | | |
| 0·60 | — | — | 0·60 | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 0·60 | — | 0·1 | 0·51 | 14 | * | 0·60 | — | 0·1 | 0·50 | 16 | * | 0·60 | — | 1·0 | 0·53 | 11 |
| 0·60 | — | 0·3 | 0·33 | 36 | † | 0·60 | — | 0·3 | 0·46 | 23 | * | 0·60 | — | 3·0 | 0·46 | 23 |
| 0·60 | — | 0·5 | 0·20 | 65 | ‡ | 0·60 | — | 0·5 | 0·38 | 36 | * | 0·60 | — | 5·0 | 0·36 | 40 |
| 0·60 | — | 1·0 | 0·05 | 92 | ‡ | 0·60 | — | 1·0 | 0·28 | 52 | * | 0·60 | — | 10·0 | 0·34 | 43 |
| 0·60 | 2·0 | — | 0·60 | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 0·60 | 2·0 | 0·1 | 0·57 | 5 | — | 0·60 | 2·0 | 0·1 | 0·57 | 5 | — | 0·60 | 2·0 | 1·0 | 0·59 | 1 |
| 0·60 | 2·0 | 0·3 | 0·45 | 25 | — | 0·60 | 2·0 | 0·3 | 0·52 | 13 | — | 0·60 | 2·0 | 3·0 | 0·59 | 1 |
| 0·60 | 2·0 | 0·5 | 0·30 | 50 | * | 0·60 | 2·0 | 0·5 | 0·41 | 31 | — | 0·60 | 2·0 | 5·0 | 0·55 | 8 |
| 0·60 | 2·0 | 1·0 | 0·08 | 86 | † | 0·60 | 2·0 | 1·0 | 0·33 | 45 | * | 0·60 | 2·0 | 10·0 | 0·53 | 11 |
| 0·60 | 4·0 | 0·1 | 0·59 | 1 | — | 0·60 | 4·0 | 0·1 | 0·59 | 1 | — | 0·60 | 4·0 | 1·0 | 0·59 | 1 |
| 0·60 | 4·0 | 0·3 | 0·52 | 13 | — | 0·60 | 4·0 | 0·3 | 0·55 | 8 | — | 0·60 | 4·0 | 3·0 | 0·59 | 1 |
| 0·60 | 4·0 | 0·5 | 0·41 | 31 | — | 0·60 | 4·0 | 0·5 | 0·51 | 15 | — | 0·60 | 4·0 | 5·0 | 0·58 | 3 |
| 0·60 | 4·0 | 1·0 | 0·18 | 72 | * | 0·60 | 4·0 | 1·0 | 0·37 | 38 | — | 0·60 | 4·0 | 10·0 | 0·58 | 3 |

* Light yellow-coloured solution. † Yellow-coloured solution. ‡ Brown red to red solution—indicated colloidal state.

livers of the injected rats. Sollmann [1936] states that reduction of tellurium compounds occurs with all living cells and that the reduced tellurium is particularly abundant in the liver and kidneys, while the lungs, spleen and adrenals are relatively free. The liver and kidneys contain appreciable amounts of cysteine, which reduces the tellurate to the colloidal state even in the presence of vitamin C.

Qualitative tests have indicated that the sulphydryl compounds, such as cysteine and glutathione, inhibit the reduction of silver nitrate [Svirbely, 1935] and of selenious acid [Emmerie, 1934] to the colloidal state by ascorbic acid. A quantitative study of these reductions by various salts was made by means of the indophenol titration method. Sulphydryl compounds were first added to the vitamin, then the salt, and, after a definite time interval (the solution being exposed to air in a 50 ml. beaker), titration was carried out rapidly in the presence of a few drops of 8 % acetic acid. The average results of three titrations, correction being made for any reduction of the dye by the sulphydryl compounds, are given in Table III. The salts themselves did not interfere with the titration.

The amounts of ascorbic acid removed by selenium dioxide, sodium selenate and tellurate paralleled the order of toxicity of the salts. Reduction of the salts (except the tellurate) to the colloidal state depended on the time interval elapsing before titration. Inhibition of this reduction in a 2 hr. interval was afforded by glutathione, cysteine (2 mg.) or a mixture of glutathione and cysteine (2 mg. each). Little protection to vitamin C was given by these sulphydryl compounds in the presence of the higher concentrations of selenium dioxide or sodium selenate, though protection was obvious with the lower concentrations and with sodium tellurate. No consistent titration values were obtained with larger amounts of cysteine since cysteine itself reduces the indophenol indicator.

The appreciable decrease in vitamin C with 1 mg. selenium dioxide, even when glutathione inhibited the assumption by the salt of the colloidal state, is of interest. Complete protection of ascorbic acid in a 2 hr. interval (estimated by the titration method) was afforded by these sulphydryl compounds (2 mg. cysteine or glutathione) in the presence of 1 mg. silver or uranyl nitrate. These salts, in the absence of cysteine or glutathione, produced a noticeable decrease in the amount of vitamin C, the percentage decreases (2 hr. interval) being 92 and 21 respectively. In confirmation of the work of Mawson [1935], it was found that the oxidation of the vitamin by 0·15 mg. Cu (as sulphate) was markedly decreased by glutathione. Such a variation in the ability to protect the vitamin, associated with inhibition of either the reduction of these metallic salts to the colloidal state or of the oxidation of the vitamin, may be explained by the formation of a stable complex of the sulphydryl compounds with the cations in the case of the heavy metals. With selenium salts, according to Bersin [1935], oxidized glutathione is formed, which may, in the glutathione-ascorbic acid system, lead to oxidation of the vitamin both *in vivo* and *in vitro*.

## Summary

1. Rats with symptoms of selenium and tellurium poisoning show marked decreases in the vitamin C content of the liver and adrenals.

2. The production of oxidized glutathione by selenium salts, in contrast to the stable complex which sulphydryl compounds form with the cations of heavy metals, may account for the decrease in ascorbic acid which is observed *in vitro* even when there is an inhibition of the reduction of the selenium to the colloidal state.

3. Selenium salts are reduced more readily by ascorbic acid than by glutathione or cysteine, but the reverse relationship holds true for sodium tellurate.

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# LXII. THE RELATION OF VITAMIN $B_1$ TO COCARBOXYLASE

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Lohmann [1937] and Lohmann & Schuster [1937] have shown that cocarboxylase is the pyrophosphate of vitamin $B_1$. They reported that the respiration of brain slices from polyneuritic pigeons could be restored to normal by *in vitro* additions of the phosphorylated vitamin. Peters [1937] has reported that cocarboxylase was only about 10% as effective as the free vitamin in similar *in vitro* experiments.

A problem of fundamental importance is the determination of the nature of vitamin $B_1$ as it occurs in living tissues, i.e. does the compound occur as the free vitamin, as the pyrophosphate, or is there a dynamic equilibrium between the two compounds?

During the course of the development of a method for the determination of the cocarboxylase content of tissues we have arrived at the conclusion that the two compounds may exist in dynamic equilibrium in living organisms, and have collected some data which relate not only to the mechanism of the phosphorylation of vitamin $B_1$ but which in a broader sense must have considerable bearing on the whole question of phosphate transfer and carbohydrate metabolism in living organisms.

As far as we are aware there is at present no direct evidence in the literature that cocarboxylase exists as such in animal tissues. Perhaps the best indication is the indirect evidence of Tauber [1937] who has reported a synthesis of cocarboxylase from vitamin $B_1$ by an enzyme prepared from duodenal mucosa. Auhagen [1933] and Simola [1932] have both reported on the cocarboxylase content of animal tissue extract, but, for reasons explained below, it now appears possible that their results could have been obtained in the complete absence of cocarboxylase from the original tissue. Their method involved the addition of a boiled tissue extract to a washed dried yeast preparation (atiozymase) and the measurement of the $CO_2$ evolved in the presence of magnesium, glucose, cozymase, acetaldehyde, hexosediphosphate and a phosphate buffer. When we attempted to determine the cocarboxylase content of boiled tissue extracts by means of the simplified system described by Lohmann (atiozymase, magnesium and pyruvate), we found that the addition of crystalline vitamin $B_1$ to the system resulted in a synthesis of cocarboxylase if a boiled tissue extract were also present. This method therefore did not distinguish between the free vitamin and the vitamin pyrophosphate but merely measured the sum of the two in the boiled extract. Thus it does not seem valid to conclude from the results of Auhagen and of Simola that cocarboxylase does in fact exist as such in animal tissues. The validity of Auhagen's demonstration of cocarboxylase in partially purified

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boiled tissue extracts is dependent upon the amount of vitamin $B_1$ and phosphorylating agents which they contained. The more recent work of Lipmann [1937] showed that cocarboxylase was necessary for the oxidation of pyruvic acid by acetone-washed *B. delbrückii*. Vitamin $B_1$ was without activity but boiled tissue extracts catalysed the reaction. Before it can be concluded that these results prove that cocarboxylase was present in the tissue extracts, it would seem desirable to show that the *B. delbrückii* preparation supplemented by the extracts is unable to carry out the synthesis which we have demonstrated to occur with the yeast preparation.

The present paper deals with the factors concerned with the phosphorylation of vitamin $B_1$ in the presence of atiozymase and boiled tissue extracts, together with a method for differentiating between the phosphorylated and the unphosphorylated vitamin in tissue extracts.

## Experimental

*Methods and apparatus.* Cocarboxylase activity was measured in terms of the rate of $CO_2$ evolved, using an atiozymase preparation with sodium pyruvate as substrate and magnesium as activator [Lohmann, 1937]. $CO_2$ output was measured by means of the Barcroft differential respirometer [Dixon, 1934]. In these experiments considerable acetaldehyde is formed which would be expected to have some effect on the manometer readings. We have checked this point by placing semicarbazide at *p*H 7·0 in the inner cup. The manometric readings were affected but slightly. Since the $O_2$ uptake was also demonstrated to be practically negligible, uncorrected readings were taken for $CO_2$ production. The experiments were carried out with air in the gas space. The flasks were shaken at 100–120 oscillations per minute at a temperature of 30°. The measurements were continued for from 1 to 2 hr. and the taps were opened momentarily when necessary.

Tissue extracts were conveniently prepared by homogenizing the tissue in an all-glass device which has been described in detail by Potter & Elvehjem [1936]. The use of this device greatly expedites the grinding of the tissue and ensures complete cell disruption.

*Enzyme.* The atiozymase employed in these experiments was prepared from brewer's yeast by the method of Auhagen [1933]. The yeast was spread in shallow pans and was dried for 3 hr. at room temperature with the aid of a fan. This preparation is stable for months when stored in a desiccator. 20 g. dried yeast were washed twice for half an hour with 700 ml. water and centrifuged. The yeast was then taken up in 700 ml. *M*/10 $Na_2HPO_4$ buffer, *p*H 7·9, and was further washed three times for 20 min. A short final washing with water followed, in order to remove the alkaline phosphate. The washed yeast (about 10 g.) was then spread on a glass plate and dried rapidly before a fan. This atiozymase preparation was not completely stable, and it was found advisable to prepare it weekly. The preparation was homogenized in *M*/10 Na and K phosphate buffer at *p*H 6·2 for use as atiozymase.

Atiozymase prepared in this manner is not completely free from cocarboxylase, but shows a great increase in activity when boiled yeast or tissue juice is added. It was also found that different atiozymase preparations, made in the same manner, show different activities. It is therefore necessary to include a manometer containing only the atiozymase, magnesium and pyruvate as a control.

*Boiled tissue extracts.* Boiled tissue extracts were prepared as follows. A chick was killed by decapitation; the liver was removed, blotted free from blood

on a filter paper, dissected free from connective tissue and placed in a weighed homogenizer tube containing 2 ml. water. The tissue was weighed, homogenized and diluted to a concentration of 333 mg. per ml. with more water. It was then plunged into a boiling water bath for 7 min. The interval between the death of the animal and the introduction of the homogenized tissue into the boiling water was 6–9 min.

*Chemical preparations.* Pyruvic acid was prepared by double distillation of commercial pyruvic acid at 2–4 mm. Hg and 38–41°. Hexosediphosphate was prepared by the method of Robison & Morgan [1930]. The insoluble barium salt was treated with the calculated amount of saturated sodium sulphate for 2 hr., and the barium sulphate was centrifuged down. The solution of the sodium hexosediphosphate was evaporated to dryness *in vacuo* and the salt was stored in a desiccator. Magnesium was added as the chloride, fluoride and iodoacetate in the form of the sodium salts. The buffer was *M*/10 Na and K phosphate, *p*H 6·2. Winthrop's synthetic vitamin $B_1$ was employed throughout.

*Procedure.* 0·4 ml. atiozymase preparation, equivalent to 100 mg. of dry preparation, was made up to 2·0 ml. with the other solutions. 0·5 ml. boiled tissue extract, corresponding to 167 mg. fresh tissue, 0·1 mg. Mg as the chloride, 10 mg. hexosediphosphate in buffer, 10 mg. Na pyruvate in buffer and 5$\gamma$ vitamin $B_1$ were employed, unless otherwise stated.

### (1) *The synthesis of cocarboxylase from vitamin $B_1$*

When boiled tissue extract was added to atiozymase plus pyruvate there was a marked increase in the $CO_2$ output. However, it was found that when vitamin $B_1$ was added to the system a still further increase in $CO_2$ evolution occurred, indicating that the added vitamin $B_1$ was synthesized to cocarboxylase. The results are shown in Table I. This experiment was repeated a number of times

Table I. *The synthesis of cocarboxylase from vitamin $B_1$ in the presence of boiled tissue extract and atiozymase*

| | Exp. A | | Exp. B | | | | |
|---|---|---|---|---|---|---|---|
| | | | Normal liver | | | Avitaminous liver | |
| Atiozymase (250 mg./ml.) (ml.) | 0·4 | 0·4 | 0·4 | 0·4 | 0·4 | 0·4 | 0·4 |
| Mg (1 mg./ml.) (ml.) | 0·1 | 0·1 | 0·1 | 0·1 | 0·1 | 0·1 | 0·1 |
| Na pyruvate (30 mg./ml.) (ml.) | 0·3 | 0·3 | 0·3 | 0·3 | 0·3 | 0·3 | 0·3 |
| Boiled liver extract (333 mg./ml.) (ml.) | — | — | — | 0·5 | 0·5 | 0·5 | 0·5 |
| Vitamin $B_1$ (50$\gamma$/ml.) (ml.) | — | 0·1 | — | — | 0·1 | — | 0·1 |
| Phosphate buffer (ml.) | 1·2 | 1·1 | 1·2 | 0·7 | 0·6 | 0·7 | 0·6 |
| $CO_2$ $\mu$l./hr. | 87·0 | 86·8 | 117·5 | 319 | 430 | 258 | 368 |

with liver and brain extracts from both normal and polyneuritic birds, and the synthesis with the avitaminous tissue extracts was usually greater. Other tissues have not as yet been used. The results in Table I show clearly that atiozymase alone is unable to convert vitamin $B_1$ into cocarboxylase, but that in the presence of boiled tissue extract the synthesis is effected. It seems likely that if the synthesis of cocarboxylase from vitamin $B_1$ occurs with atiozymase and boiled tissue extracts in the presence of pyruvate, it will certainly occur with atiozymase and boiled tissue extracts in the presence of glucose, hexosediphosphate, cozymase and acetaldehyde. From this it is apparent that any vitamin $B_1$

present in the tissue extract will be converted into cocarboxylase in the presence of the atiozymase, and that the method used by Auhagen and by Simola does not differentiate between the free vitamin and cocarboxylase in boiled tissue extracts.

It was clear that one or more thermostable compounds present in the boiled tissue extract were necessary for the phosphorylation of the free vitamin in the presence of atiozymase. Our attention was next turned to the identification of these constituents. The following compounds were considered to be the likely constituents of the boiled tissue extract: hexosediphosphate, cozymase, adenylic acid and adenylpyrphosphate.

### (2) *The effect of hexosediphosphate*

In attempting to compare the cocarboxylase contents of normal and avitaminous boiled liver extracts, using simply atiozymase, magnesium, pyruvate and extract, we found that the livers from polyneuritic birds sometimes gave higher values than those from normal birds. Simola had employed hexosediphosphate in all his experiments and had found significant differences between normal and avitaminous tissues. Furthermore, it had been demonstrated by Nagai [1932] that there was a large increase in the hexosediphosphate content of all tissues, except brain, from avitaminous birds. We therefore added hexosediphosphate to the system and found a large increase in $CO_2$ output (Table II) which was greater with normal than with avitaminous tissues, in confirmation of the results of Simola. However, it must be remembered that these results indicate the sum of free vitamin and cocarboxylase.

Table II. *The stimulating effect of hexosediphosphate upon the synthesis of cocarboxylase in the presence of boiled tissue extract with and without added vitamin $B_1$*

| | Normal liver | | | | Avitaminous liver | | |
|---|---|---|---|---|---|---|---|
| Vitamin $B_1$ (50 $\gamma$/ml.) (ml.) | — | — | — | 0·1 | — | — | 0·1 |
| Hexosediphosphate (50 mg./ml.) (ml.) | — | — | 0·2 | 0·2 | — | 0·2 | 0·2 |
| Boiled liver extract (333 mg./ml.) (ml.) | — | 0·5 | 0·5 | 0·5 | 0·5 | 0·5 | 0·5 |
| Buffer (ml.) | 1·2 | 0·7 | 0·5 | 0·4 | 0·7 | 0·5 | 0·4 |
| $CO_2$ $\mu$l./2 hr. | 144 | 316 | 1100 | 1522 | 255 | 500 | 1353 |

Standard quantities of atiozymase, Mg and pyruvate were present in every flask. See Table I.

The stimulating effect of hexosediphosphate upon the $CO_2$ output could be explained by postulating that hexosediphosphate acted as a phosphate donator in the conversion of vitamin $B_1$ into cocarboxylase. This "phosphate-donating" action might be direct, or indirect. If indirect, several possibilities would have to be considered. The phosphate might be transferred to cozymase [Lennerstrand, 1936] or to adenylic acid, or the hexosediphosphate might break down to phosphopyruvate which could serve as the phosphate donator; the hexosediphosphate might yield phosphoglyceric acid to give energy for the esterification of inorganic phosphate. Further experiments were carried out to check these possibilities.

In order to check the possibility that the transfer was direct, we determined whether or not hexosediphosphate was the only significant thermostable component which the boiled extract added. This was done by comparing the synthesizing powers of the system atiozymase, magnesium, pyruvate, vitamin $B_1$ and hexosediphosphate, with and without added boiled tissue extract. A small

though definite synthesis was observed without the tissue extract, but the extent of this synthesis was greatly increased by the addition of the extract (Table III). Von Euler & Vestin [1937] had reported synthesis with hexosediphosphate or adenosinetriphosphate and vitamin $B_1$ added to an unwashed yeast

Table III. *The stimulating effect of boiled liver extract upon the synthesis of cocarboxylase from vitamin $B_1$ in the presence of hexosediphosphate*

| | Exp. A | | | | Exp. B | | | |
|---|---|---|---|---|---|---|---|---|
| Vitamin $B_1$ (50 $\gamma$/ml.) (ml.) | — | 0·1 | — | 0·1 | — | 0·1 | — | 0·1 |
| Hexosediphosphate (50 mg./ml.) (ml.) | — | — | 0·2 | 0·2 | — | — | 0·2 | 0·2 |
| Boiled liver extract (333 mg./ml.) (ml.) | — | — | — | — | 0·5 | 0·5 | 0·5 | 0·5 |
| Buffer (ml.) | 1·2 | 1·1 | 1·0 | 0·9 | 0·7 | 0·6 | 0·5 | 0·4 |
| $CO_2$ $\mu$l./hr. | 126 | 149 | 150 | 186 | 232 | 296 | 817 | 1243 |

Standard quantities of atiozymase, Mg and pyruvate were present in every flask. See Table I.

preparation. Consequently, we repeated our experiments using boiled yeast juice and obtained the same results as were obtained with the tissue extracts. It was readily apparent, therefore, that hexosediphosphate alone was not sufficient to produce the synthesis and that the effect of boiled tissue extracts was due to factors other than hexosediphosphate. It may be concluded that the phosphorylation of the vitamin does not occur as a result of a direct transfer of phosphorus from hexosediphosphate.

### (3) *The effect of fluoride*

Another explanation of the hexosediphosphate effect might lie in the ability of phosphopyruvic acid to act as a phosphate donator [Needham & Lehmann, 1937]. Hexosediphosphate might be expected to break down via triosephosphate and phosphoglyceric acid to phosphopyruvic acid. If the latter were the source of phosphate, the hexosediphosphate effect should be abolished by the action of fluoride.

Sodium fluoride in various concentrations was added to the system in an attempt to inhibit the synthesis. The complete system of atiozymase, magnesium, pyruvate, hexosediphosphate, vitamin $B_1$ and boiled tissue extract was employed. The results are shown in Table IV and Fig. 1.

Table IV. *The effect of graded molarities of sodium fluoride upon the synthesis of cocarboxylase in the presence of hexosediphosphate, vitamin $B_1$ and boiled liver extract*

| | Exp. A | | | | | | Exp. B | |
|---|---|---|---|---|---|---|---|---|
| Vitamin $B_1$ (50 $\gamma$/ml.) (ml.) | — | 0·1 | 0·1 | 0·1 | 0·1 | 0·1 | — | — |
| Hexosediphosphate (50 mg./ml.) (ml.) | — | 0·2 | 0·2 | 0·2 | 0·2 | 0·2 | — | — |
| Boiled liver extract (333 mg./ml.) (ml.) | 0·5 | 0·5 | 0·5 | 0·5 | 0·5 | 0·5 | — | — |
| Na fluoride (0·4 *M*) (ml.) | — | — | 0·2 | 0·1 | — | — | — | 0·2 |
| Na fluoride (0·1 *M*) (ml.) | — | — | — | — | 0·2 | 0·1 | — | — |
| Buffer (ml.) | 0·7 | 0·4 | 0·2 | 0·3 | 0·2 | 0·3 | 1·2 | 1·0 |
| $CO_2$ $\mu$l./2 hr. | 356 | 2012 | 803 | 1047 | 968 | 1055 | 207 | 212 |

Standard quantities of atiozymase, Mg and pyruvate were present in every flask. See Table I.

The experiments with added fluoride clearly indicate that the synthesis of cocarboxylase is only partially checked by the inhibition of the reaction by

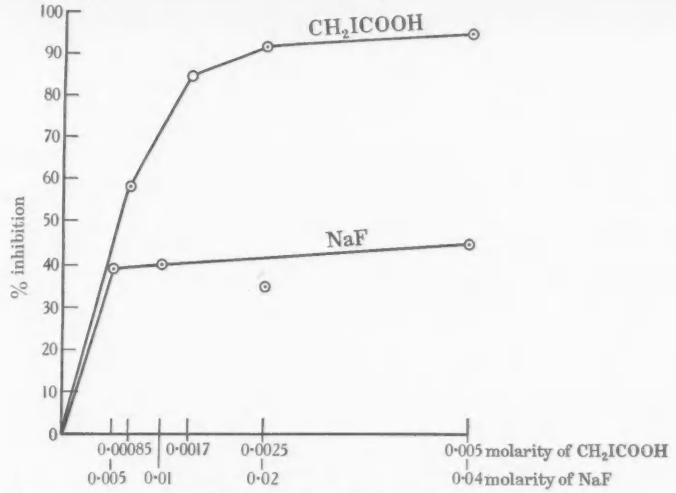


Fig. 1. The effect of various concentrations of iodoacetic acid and sodium fluoride on the phosphorylation of vitamin $B_1$. For experimental conditions see Tables IV and V.

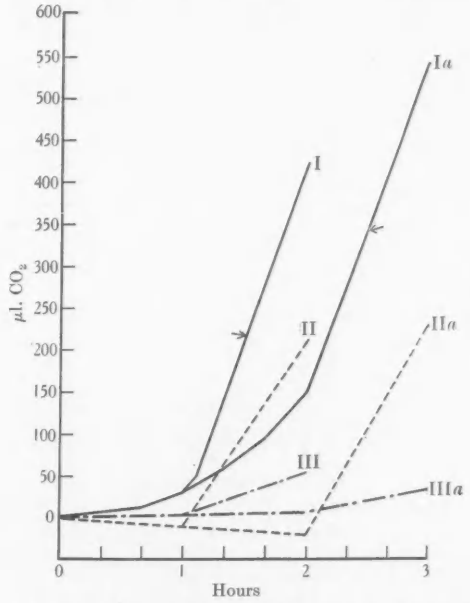


Fig. 2. The effects of sodium fluoride and iodoacetic acid on the synthesis and action of cocarboxylase. ——— Control. Curves I and I*a*. - - - - 0·04 *M* NaF from the beginning. Curves II and II*a*. –·–·–·– 0·005 *M* $CH_2ICOOH$ from the beginning. Curves III and III*a*. 10 mg. pyruvate added to I, II and III at 1 hr. 10 mg. pyruvate added to I*a*, II*a*, and III*a* at 2 hr. $CH_2ICOOH$ to make 0·005 *M* added to I and I*a* 30 min. after pyruvate addition, as shown by arrows. All flasks contained atiozymase, hexosediphosphate, boiled liver extract, magnesium, vitamin $B_1$ and phosphate buffer from the beginning.

which phosphopyruvic acid is formed. There appears to be a lag in the evolution of $CO_2$ when fluoride is used, and it is possible that there are two mechanisms for phosphorylating the vitamin, and that while fluoride blocks the reaction involving phosphopyruvate, it has no effect on the other mechanism.

In a further attempt to demonstrate a breakdown of hexosediphosphate to pyruvic acid an experiment was carried out omitting the addition of pyruvate. The results are shown in Fig. 2 (before pyruvate additions). The amount of $CO_2$ evolved before the addition of pyruvate was very small, indicating that little pyruvate was formed. However, it is possible that phosphopyruvic acid was formed in considerable amounts and was dephosphorylated only to the small extent necessary for the synthesis of cocarboxylase.

### (4) *The effect of iodoacetic acid*

Since it appeared that hexosediphosphate might be effecting the synthesis by a mechanism other than that involving phosphopyruvate, we decided to test the effect of iodoacetate. Needham & Pillai [1937] have furnished evidence for the coupling of oxido-reductions with the esterification of inorganic phosphate and have shown that adenylpyrophosphate can be formed from adenylic acid and inorganic phosphate when energy is obtained from the conversion of triose-phosphate into phosphoglyceric acid. It was believed that the reactions were coupled by means of cozymase. The reaction was prevented by iodoacetate which acted by inhibiting the energy-yielding oxido-reductions.

When iodoacetate was added to the system which contained all the components necessary for the synthesis of cocarboxylase, it was found that the synthetic reaction was virtually abolished. These results are shown in Table V

Table V. *The effect of graded molarities of iodoacetic acid upon the synthesis of cocarboxylase by atiozymase in the presence of hexosediphosphate, vitamin $B_1$ and boiled liver extract*

| | Exp. A | | | | | | Exp. B | |
|---|---|---|---|---|---|---|---|---|
| Vitamin $B_1$ (50 $\gamma$/ml.) (ml.) | 0·1 | — | 0·1 | 0·1 | 0·1 | 0·1 | — | — |
| Hexosediphosphate (50 mg./ml.) (ml.) | 0·2 | — | 0·2 | 0·2 | 0·2 | 0·2 | — | — |
| Boiled liver extract (333 mg./ml.) (ml.) | 0·5 | 0·5 | 0·5 | 0·5 | 0·5 | 0·5 | — | — |
| Na iodoacetate (0·05 *M*) (ml.) | — | — | 0·2 | 0·1 | — | — | — | 0·2 |
| Na iodoacetate (0·017 *M*) (ml.) | — | — | — | — | 0·2 | 0·1 | — | — |
| Buffer (ml.) | 0·4 | 0·7 | 0·2 | 0·3 | 0·2 | 0·3 | 1·2 | 1·0 |
| $CO_2$ $\mu$l./2 hr. | 1828 | 425 | 465 | 528 | 590 | 1017 | 207 | 200 |

Standard quantities of atiozymase, Mg and pyruvate were present in every flask. See Table I.

and Fig. 1. Both fluoride and iodoacetate were without effect on cocarboxylase action *per se* as was shown by the complete absence of inhibition of the atiozymase preparation. The effects of fluoride and iodoacetate upon the synthesis of cocarboxylase and upon its action were further demonstrated in the following experiment.

Atiozymase, boiled tissue extract, hexosediphosphate, magnesium and vitamin $B_1$ in phosphate buffer were shaken in Barcroft vessels in the absence of pyruvate, with and without inhibitors; $CO_2$ production was measured throughout the experiment. At the end of 1 hr. the manometers were disconnected long enough to introduce pyruvate into all the flasks and iodoacetate in a Keilin dangler into the control flasks. After equilibration, the cocks were closed and the

$CO_2$ evolution was again measured for 20 min. The Keilin dangler was then shaken down in the control flasks and the measurement of $CO_2$ evolution was continued. A parallel run was made in which the pyruvate was added at 2 hr. The results are shown in Fig. 2. In the absence of pyruvate a small amount of $CO_2$ was evolved at an increasing rate from the control flask, indicating that slight amounts of pyruvate were being formed. The presence of either fluoride or iodoacetate inhibited this $CO_2$ output. When pyruvate was added, there was a rapid evolution of $CO_2$ from the control flask and from the flask which contained fluoride, indicating that cocarboxylase had been synthesized in these two flasks. In the flask containing iodoacetate there was relatively little $CO_2$ evolved, indicating that the synthesis was inhibited by the iodoacetate. The slight evolution of $CO_2$ which did occur can be attributed to the small amounts of cocarboxylase originally present in the atiozymase. The flask which contained fluoride throughout demonstrated that fluoride inhibited neither the synthesis nor the action of cocarboxylase. The flask which contained iodoacetate throughout showed that the compound inhibited the synthesis of cocarboxylase, and the fact that the delayed addition of iodoacetate to the control flask caused no diminution in the rate of $CO_2$ output clearly demonstrated that the poison had no effect on the action of preformed cocarboxylase.

The inhibitory action of iodoacetate makes it appear likely that an oxido-reduction is involved in the synthesis of cocarboxylase. The reactions described by Needham & Pillai [1937] were all dismutations and did not require oxygen. Our experiments have all been carried out in the presence of air and in addition effective shaking has always been secured. The $O_2$ uptake is however very small, and is certainly too small to have much effect on the volume of $CO_2$

Table VI. *The $O_2$ uptake and $CO_2$ production during the enzymic synthesis of cocarboxylase*

| | | | | | | |
|---|---|---|---|---|---|---|
| Vitamin $B_1$ (50 $\gamma$/ml.) (ml.) | — | — | 0·1 | 0·1 | 0·1 | 0·1 |
| Hexosediphosphate (50 mg./ml.) (ml.) | — | — | 0·2 | 0·2 | 0·2 | 0·2 |
| Boiled liver extract (333 mg./ml.) (ml.) | 0·5 | 0·5 | 0·5 | 0·5 | 0·5 | 0·5 |
| Na iodoacetate (0·05 *M*) (ml.) | — | — | — | — | 0·2 | 0·2 |
| Buffer (ml.) | 0·7 | 0·7 | 0·4 | 0·4 | 0·2 | 0·2 |
| $CO_2$ $\mu$l./2 hr. | 153 | — | 782 | — | 200 | — |
| $O_2$ $\mu$l./2 hr. | — | 8·1 | — | −24·5 | — | 2·7 |

Standard quantities of atiozymase, Mg and pyruvate were present in every flask. See Table I.

evolved (Table VI). If the synthesis involves a dismutation between triosephosphate and pyruvate to form phosphoglycerate and lactate, the amount of lactate formed would be very small in comparison with the amount of pyruvate present. The excess pyruvate would be sufficient to inhibit the oxidation of the lactate [Green & Brosteaux, 1936].

Lipmann [1937] has shown that cocarboxylase is required for the oxidative decarboxylation of pyruvic acid by acetone-treated *B. delbrückii*. In this reaction pyruvic acid is dehydrogenated and oxygen is taken up. He has found that flavin phosphate speeds up the reaction. It seems likely that this compound is involved in a hydrogen transport mechanism and there is little reason to believe that it is involved in the simple decarboxylation of pyruvic acid by atiozymase together with cocarboxylase.

The experiments detailed above suggest that the difficulties involved in Simola's measurements might be overcome by the proper use of iodoacetate so as to inhibit the conversion of vitamin $B_1$ into cocarboxylase. Table VII indicates the type of experiment by which this may be done. In order to determine

Table VII. *The relative vitamin $B_1$ and cocarboxylase contents of boiled normal liver extract*

| | | | | | |
|---|---|---|---|---|---|
| Vitamin $B_1$ (10 $\gamma$/ml.) (ml.) | — | — | — | — | 0·2 |
| Hexosediphosphate (50 mg./ml.) (ml.) | — | — | — | 0·2 | 0·2 |
| Boiled tissue extract (333 mg./ml.) (ml.) | — | — | 0·5 | 0·5 | 0·5 |
| Na iodoacetate (0·05 *M*) (ml.) | — | 0·2 | 0·2 | — | — |
| Buffer (ml.) | 1·2 | 1·0 | 0·5 | 0·5 | 0·3 |
| $CO_2$ $\mu$l./2 hr. | 189 | 189 | 265 | 1885 | 2154 |

Standard quantities of atiozymase, Mg and pyruvate were present in every flask. See Table I.

whether the boiled extract contains vitamin $B_1$ or cocarboxylase it is supplemented with hexosediphosphate to give the combined effect of the vitamin and the vitamin pyrophosphate and is poisoned with iodoacetate to measure the vitamin pyrophosphate alone. The results in Table VII indicate that by far the greater amount of the vitamin is in the unphosphorylated form in the boiled extract from normal chick liver. It remains to be shown how closely this approximates to the situation *in vivo*. In a later paper we expect to present data on this point together with the results of an investigation on the relative amounts of the two compounds in the various tissues of normal and avitaminous animals.

## Discussion

Although many experiments need to be done to check all the questions raised by the data in the section above, we feel that certain possibilities are definitely indicated regarding the mechanism of the phosphorylation of vitamin $B_1$.

There seem to be reasons for believing that the vitamin may be phosphorylated either by adenylpyrophosphate or by phosphorylated cozymase. There does not at present appear to be sufficient evidence in the literature to differentiate clearly between the phosphorylating abilities of these two compounds, and we will not attempt to distinguish between them here. It seems fairly definite that phosphorylated cozymase can form adenylpyrophosphate. In this discussion we shall consider the evidence relating to the phosphorylation of cozymase, while at the same time we recognize the possibility that adenylpyrophosphate may be the compound which ultimately completes the synthesis.

Before proceeding further it should be stated that we have no evidence to disprove the possibility that the vitamin can be phosphorylated directly by the various mechanisms which can phosphorylate adenylic acid or cozymase. The recent paper by Needham & Pillai [1937] indicates that the phosphorylation of cozymase by inorganic phosphate can be coupled with certain dismutations. The fact that the thiazole ring in vitamin $B_1$ contains a bond capable of oxidation and reduction [Lipmann, 1936] may be a further indication of an analogy between the phosphorylations of the vitamin and of cozymase.

We may now proceed to a consideration of the mechanisms which may be involved in the preliminary phosphorylation of cozymase or adenylic acid.

Ohlmeyer [1935] reported that in the presence of Lebedev yeast extract, hexosediphosphate could phosphorylate adenylic acid to yield hexosemonophosphate and adenylpyrophosphate. The reaction could be inhibited by iodoacetate. This reaction must be considered to provide a possible explanation for our results until experiments with phosphoglyceraldehyde can be done. However, Needham & Pillai were unable to find any support for the occurrence of this reaction with muscle extract.

Needham & Pillai showed that the dismutation of triosephosphate could yield energy for the phosphorylation of adenylic acid by inorganic phosphate via cozymase. This reaction was inhibited by iodoacetate and appears to us to be a likely explanation for one of the mechanisms concerned in the synthesis of cocarboxylase. The dependence of cocarboxylase upon inorganic phosphate [Lipmann, 1937] also seems to support this idea.

The possibility remains that phosphopyruvic acid can be the source of phosphate, although it has been definitely ruled out as the only mechanism, since the vitamin can be phosphorylated in the presence of fluoride, which is known effectively to prevent the conversion of phosphoglyceric into phosphopyruvic acid. It would be desirable to attempt the synthesis of cocarboxylase using phosphopyruvic acid with and without any accessory phosphate carrier.

It is interesting to note that Birch & Mapson [1936] attributed the bradycardia associated with polyneuritis to an increased amount of adenylic acid in the heart, while Nagai [1932] found a decrease in adenylpyrophosphate in polyneuritis. These observations suggest the possibility that cocarboxylase might even be acting as a phosphate donator to adenylic acid rather than that the free vitamin accepts phosphate from adenylpyrophosphate.

In the experiments described in this paper, the synthesis of cocarboxylase was effected by yeast enzyme. The close analogy which is known to exist between yeast and animal phosphorylations would make it seem likely that the vitamin is phosphorylated in a similar manner in yeast and in animal tissues. That it is phosphorylated in the former is indicated by our work and in the latter (in duodenal mucosa) by the work of Tauber [1937]. It remains to be seen whether or not the synthesis can be effected in the presence of the enzymes of muscle extract. If this proves to be the case the present schemes for glycolysis will have to be revised to take into account one more compound which exists in a phosphorylated and in an unphosphorylated state.

## Summary

1. The synthesis of cocarboxylase from vitamin $B_1$ has been shown to take place in the presence of washed dried yeast, hexosediphosphate and boiled tissue extract.

2. The synthesis of cocarboxylase is almost completely inhibited by $0{\cdot}005\,M$ iodoacetic acid, but is only slightly inhibited by $0{\cdot}04\,M$ sodium fluoride. Neither of the inhibitors affects the action of preformed cocarboxylase.

3. By the proper combination of inhibitors, activators and enzyme, it is possible to differentiate between cocarboxylase and vitamin $B_1$ in boiled tissue extracts. The presence of both compounds has been demonstrated. It is believed that the two compounds may exist in dynamic equilibrium *in vivo*.

4. The possible mechanisms for the synthesis of cocarboxylase from vitamin $B_1$ are discussed in the light of the data presented.

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# LXIII. VITAMIN K IN THE PLANT

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Previous investigations have dealt with the occurrence and fate of vitamin K in the plant [Dam, 1934, 1935; Dam & Schønheyder, 1936; Almquist & Stokstad, 1935; Almquist, 1937]; in this paper we have recorded the results of a more detailed study of the problem. We first determined the vitamin in flowers, seeds and roots from green plants and in certain lower plants, and then studied the distribution of the vitamin in relation to the influence of light and darkness on the germinating plant, the withering process occurring in leaves and the well-known degeneration which leads to the formation of chlorophyll-free areas on leaves.

*Technique.* The plant materials were in most cases dried in vacuum desiccators at room temperature in the laboratory. A few materials were supplied from a manufactory, where they had been rapidly dried by warm air.

The determinations were made by the curative test method used in this laboratory. This method, which was described by Schønheyder [1936], has been steadily developed [Dam & Lewis, 1937] during the work, and is now capable of giving reasonably accurate results. It will be described in a separate paper.

Dam & Schønheyder [1936] showed that direct feeding of dried plant material yielded somewhat lower values than feeding of the light petroleum extract, a fact which was explained by the better absorbability of the extract; the present determinations were, however, all made by direct feeding.

## Results

The following results were obtained. In all cases the figures indicate the number of units of vitamin K per g. dry material.

| | |
|---|---|
| Green leaves of | |
| Alfalfa | 200 |
| Alfalfa | 300 |
| Alfalfa | 400 |
| Cabbage | 400 |
| Spinach | 550 |
| Grass | 200 |
| Cauliflower | 400 |
| Nettle | 400 |
| Chestnut | 800 |
| Pine needles | 200 |
| Spruce needles | 120 |
| Etiolated leaves of | |
| Peas (+stem, grown in the dark) | 70 |
| Cabbage (inner leaves) | 100 |
| Leaves with chlorophyll-free areas | |
| *Codiaeum variegatum* | |
| Green parts | 300 |
| Yellow parts | 250 |
| Withering process | |
| Chestnut leaves | |
| Green | 800 |
| Yellow | 600 |
| Brown | 800 |
| Flowers of | |
| Sunflower | 20 |
| Cauliflower | 70 |
| Fruits of | |
| Strawberry, ripe | 15 |
| Hips, ripe | 40 |
| Tomato, unripe, green | 100 |
| Tomato, ripe, red | 50 |

[1] This work was aided by a grant from the Josiah Macy Jr. Foundation. The beginning of the work was aided by a grant from P. Carl Petersen's Fond.

| | |
|---|---|
| Seeds of | |
| Sunflower | Less than 10 |
| Hemp seed | 40 |
| Soy bean | 25 |
| Pea (ripe for sowing) | 14 |
| Oats | Less than 10 |
| Wheat | Less than 5 |
| Yellow corn | Less than 5 |
| Wheat bran | 10 |
| Wheat germ | Less than 5 |
| Roots and tuber bulbs | |
| Mangold | Less than 5 |
| Carrots | About 10 |
| Potatoes | Less than 10 |
| Lower plants | |
| Moss | About 50 |
| Seaweed: | |
| *Ulva lactuca* | 130 |
| *Enteromorpha clathrata* | 140 |
| *Rhodymenia palmata* | 170 |
| *Ascophyllum nodosum* | 170 |
| Lichen | About 30 |
| Fungus | |
| Common mushroom | About 10 |

Peas germinated in the light and in the dark:

| | Wt. g. | Units per g. | Units per plant |
|---|---|---|---|
| Peas before sowing | 0·2 | 14 | 2·8 |
| Peas grown in the light (275 plants) | | | |
| Plant and root, 16 days old | 0·0715 | 300 | 21·5 |
| Pea, 16 days old | 0·0335 | 35 | 1·2 |
| | | Total | 22·7 |
| Peas grown in the dark (204 plants) | | | |
| Plant and root, 16 days old | 0·0664 | 70 | 4·7 |
| Pea, 16 days old | 0·0376 | 25 | 0·9 |
| | | Total | 5·6 |

## Discussion

In accordance with previous results green leaves were found to be the most potent source of vitamin K. Among the species examined, chestnut leaves were the most potent. Seeds and roots and some fruits were rather poor sources, while other fruits—tomatoes—occupied a middle position. The outer flowers of sunflowers were rather poor sources while the cauliflower head corresponded in potency to green tomatoes and etiolated leaves (see below). Those lower plants which contained chlorophyll appeared to be somewhat poorer sources than the higher plants. The mushroom, which contains no chlorophyll, was very poor in vitamin K. It was not possible to conclude from the experiment that the fungus had synthesized the small amount of the vitamin which it contained.

Leaves of the chestnut were found to lose only a small portion, if any, of their vitamin K activity when their chlorophyll disappeared during the withering process. Yellow and brown leaves contained about 70–100 % of the vitamin found in green leaves growing on the same tree.

Leaves of the plant *Codiaeum variegatum*, which exhibit large yellow areas alternating with green ones, were found to contain almost as much vitamin K in the yellow parts as in the green, the difference being apparently about 20 % in favour of the green parts, though it may not be significant.

Leaves etiolated in the dark contained much less vitamin K than the corresponding green leaves.

The experiment with peas grown in the light and in the dark for 16 days showed that while a large amount of vitamin was synthesized in the light (about 7 times the amount originally contained in the pea from which the plant germinated) there was only a very limited synthesis in the dark.

The results for soy beans were in conformity with previous results obtained by the preventive method [Dam, 1935] which showed complete protection when 60 % soy bean meal was incorporated in the diet and haemorrhages and clotting times were used as criteria.

SUMMARY

The amount of vitamin K was determined in a series of plant organs by the curative technique.

The richest sources were green leaves of different kinds, chestnut leaves being the most potent among the species examined.

During the withering of green leaves the amount of vitamin K was not much altered.

Leaves which had large yellow areas alternating with green ones contained approximately as much vitamin K in the yellow areas as in the green.

The vitamin K content of flowers, roots and seeds was found to be much less than that of green leaves; mangold root was a particularly poor source.

Certain lower plants appeared to be poorer sources than higher plants.

An experiment with germinating peas showed that whereas there was an abundant synthesis of the vitamin in peas grown in the light, synthesis in peas grown in the dark occurred to a very limited extent.

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# LXIV. NEW ZEALAND FISH OILS

## II. SEASONAL VARIATIONS IN THE VITAMIN A CONTENT OF LING (*GENYPTERUS BLACODES*) LIVER OIL

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(*Received 11 January 1938*)

Previous studies [Shorland, 1935; 1937] have shown that the ling, in contrast to the fish examined by other workers, exhibits no major seasonal variations in the vitamin A potency of the liver oil, although the data so far obtained are insufficient to detect minor fluctuations. The object of this work is to study further the variations in the liver oil potency of vitamin A and to ascertain its distribution both in the tissues and in the food.

The livers from each batch of fish caught in Cook Straits, within 7–18 miles of Island Bay, Wellington, were placed in a number of cloth bags and autoclaved with low pressure steam [cf. Shorland, 1935]. The additional extract obtained by pressing was mixed with the "steamed" oil and the combined yield was recorded. Some of the residues from the pressing process were dried and extracted with ether to assess the total oil content.

Calculation of the vitamin A content was made on the basis of values obtained with the richest vitamin A distillates [Carr & Jewell, 1933], $E^{1\%}_{1\,\mathrm{cm.}}$ 328 m$\mu$ = 1600, Carr-Price value = 78,000, the latter being mainly used, though wherever possible the intensity of absorption at 328 m$\mu$ has been taken as the final criterion. The figures recorded here refer to the content of $C_{20}H_{29}OH$ only and other substances [cf. Morgan *et al.* 1935] possessing possible vitamin A activity were ignored. Fish livers yielding by extraction with solvents oils of high vitamin A potency sometimes gave "steamed" oils of much lower potency [cf. Schmidt-Nielson & Schmidt-Nielsen, 1929]. The "steamed", "pressed" or petrol-extracted [cf. Denz & Shorland, 1934] preparations from any given batch of ling livers showed, however, little variation in vitamin A content. In this work, therefore, the vitamin A figures recorded in Table I are conveniently based on the combined "pressed" and "steamed" extracts.

Although nearly 3000 livers have been used, the minor monthly fluctuations (cf. Table I) may not be significant but may be due to errors in sampling. In Fig. 1, therefore, the effect of this error has been minimized by taking the mean bi-monthly values. These show a progressive decrease in vitamin A content from 0·7 % in January–February to 0·5 % in July–August. The slightly diminished vitamin A content towards the end of the season, which lasts from April to September, is in accordance with the previous data [Shorland, 1935] and with measurements made on bulk samples. The latter showed a mean Carr-Price value of 490 during the earlier part of the 1936 season, but for the later samples the lower value of 450 was obtained, according to data kindly supplied by British Drug Houses, Ltd. Detailed data were not available for the period October to December, but the few samples analysed both in this and the

Table I. *Cook Strait ling liver oils*

| Date of catch | Approx. total wt. livers kg. | Mean wt. liver g. | Yield of oil % | Iodine value (Wijs 1 hr.) | Carr-Price B.U. | Vit. A content of oil from B.U. % | $E^{1\%}_{1\,cm.}$ 328 mμ | Vit. A content of oil from U.V. % | Mean liver wt. for month g. | Mean % vit. A for month |
|---|---|---|---|---|---|---|---|---|---|---|
| 16. i. 35 | 10·4 | 612 | 20·4 | 141·1 | 360 | 0·46 | 7·5 | 0·47 | | |
| 24. i. | 44·4 | 661 | 17·5 | 135·6 | 440 | 0·56 | 8·0 | 0·50 | 627 | 0·56 |
| 31. i. | 7·7 | 453 | — | 142·9 | 780 | 1·00 | 15·8 | 0·99 | | |
| 4. ii. | 3·62 | 453 | 40·6 | 144·8 | 300 | 0·38 | 6·7 | 0·42 | | |
| 13. ii. | 11·3 | 494 | 20·8 | 139·0 | 660 | 0·85 | 15·4 | 0·96 | 652 | 0·85 |
| 14. ii. | 45·3 | 721 | 32·0 | 134·6 | 790 | 1·01 | 14·6 | 0·91 | | |
| 24. ii. | 8·6 | 576 | 19·5 | 149·1 | 440 | 0·56 | 8·7 | 0·54 | | |
| 1. iii. | 27·2 | 796 | 24·7 | 138·0 | 650 | 0·83 | 11·9 | 0·74 | | |
| 6. iii. | 11·3 | 540 | 22·0 (39·4)* | 143·4 | 430 | 0·55 | 7·8 | 0·49 | 651 | 0·55 |
| 11. iii. | 27·6 | 553 | 28·6 | 139·9 | 630 | 0·81 | 12·4 | 0·78 | | |
| 23. iii. | 57·1 | — | 19·8 | 144·8 | 230 | 0·29 | 5·3 | 0·33 | | |
| 7. iv. | 25·3 | 793 | 23·0 (37·4) | 142·3 | 460 | 0·59 | 11·4 | 0·71 | | |
| 25. iv. | 11·3 | 812 | 18·8 | 149·1 | 480 | 0·62 | 7·9 | 0·49 | | |
| 25. iv. | 22·7 | — | — | 153·2 | 480 | 0·62 | 8·6 | 0·54 | 707 | 0·74 |
| 26. iv. | 11·3 | 630 | 20·0 | 143·6 | 560 | 0·72 | 11·5 | 0·72 | | |
| 27. iv. | 18·1 | 566 | 23·2 | 131·5 | 940 | 1·21 | 18·8 | 1·18 | | |
| 10. v. | 24·9 | 998 | 22·9 | 139·0 | 430 | 0·55 | 10·4 | 0·65 | | |
| 22. v. | 17·7 | 503 | — | 148·1 | 240 | 0·31 | 5·65 | 0·35 | | |
| 25. v. | 81·6 | 730 | — | 149·1 | 390 | 0·50 | 9·3 | 0·58 | | |
| 26. v. | 117·9 | 892 | 16·4 (32·8) | 140·6 | 530 | 0·68 | 10·1 | 0·63 | 798 | 0·60 |
| 27. v. | 20·4 | 417 | 23·5 | 143·3 | 500 | 0·64 | 11·1 | 0·69 | | |
| 27. v. | 24·4 | 906 | — | 143·3 | 470 | 0·60 | 8·9 | 0·56 | | |
| 2. vi. | 43·0 | — | 22·4 | — | 430 | 0·55 | 8·1 | 0·51 | | |
| 20. vi. | 36·2 | — | 28·5 | 143·2 | 450 | 0·58 | 8·95 | 0·56 | | |
| 21. vi. | 27·2 | — | 22·2 | 135·8 | 760 | 0·98 | 15·4 | 0·96 | | |
| 24. vi. | 33·1 | 621 | 21·9 (33·8) | 141·6 | 490 | 0·63 | 11·4 | 0·71 | 630 | 0·68 |
| 28. vi. | 12·7 | 634 | 27·0 (43·9) | 146·7 | 520 | 0·67 | 10·6 | 0·66 | | |
| — vi. 36 | 13·6 | 648 | — | 140·6 | 760 | 0·98 | 14·0 | 0·88 | | |
| 2. vii. 35 | 4·1 | ?034 | | 136·4 | 410 | 0·53 | 8·3 | 0·52 | | |
| 3. vii. | 163·4 | — | — | 146·2 | 430 | 0·55 | 8·8 | 0·55 | | |
| 6. vii. | 54·4 | — | — | 147·5 | 470 | 0·60 | 8·5 | 0·53 | | |
| 9. vii. | 23·5 | 739 | 21·9 (39·6) | 148·7 | 230 | 0·29 | 5·2 | 0·33 | 679† | 0·57 |
| 15. vii. | 489·5 | — | — | 145·2 | 410 | 0·53 | 9·5 | 0·59 | | |
| 18. vii. | 6·8 | 1700 | — | 138·9 | 220 | 0·28 | 5·2 | 0·33 | | |
| 22. vii. | 20·85 | 771 | 18·0 | 139·0 | 410 | 0·53 | 9·25 | 0·58 | | |
| 2. viii. | 18·1 | 648 | 19·2 | 142·2 | 230 | 0·29 | 4·0 | 0·25 | | |
| 22. viii. | 23·6 | 942 | 16·4 | 139·8 | 320 | 0·41 | 7·1 | 0·44 | 810 | 0·43 |
| 18. viii. 36 | 22·2 | 671 | 15·3 | 150·8 | 470 | 0·60 | 9·0 | 0·56 | | |
| — viii. | 24·9 | 925 | 18·2 | 139·3 | 410 | 0·53 | 7·0 | 0·44 | | |
| 4. ix. 35 | 145·0 | — | — | 142·6 | 420 | 0·54 | 8·9 | 0·56 | — | 0·56 |
| 28. ix. | 2·7 | — | — | 146·4 | 330 | 0·42 | 5·9 | 0·37 | | |
| — xi. 36 | — | — | — | 149·4 | 690 | 0·89 | 15·0 | 0·94 | — | — |
| 5. xii. | 3·6 | — | — | 141·8 | 370 | 0·47 | 8·10 | 0·51 | | |

* Figures in brackets refer to total oil content.

† The data for the liver weights are not representative, the figures given in the table are therefore calculated from 308 lb. of livers collected during July 1934 and July 1935.

preceding investigations suggest that the vitamin A content does not vary greatly from the mean monthly value of 0·615 % (Carr-Price value, 486; $E^{1\%}_{1\,cm.}$ 328 mμ=9·84). The mean amount of vitamin A in ling liver was 215 mg./100 g. tissue or 1494 mg./liver, as compared with 316·5 mg. vitamin A/100 g. tissue or 1434 mg./liver for Aberdeen samples of halibut calculated on the basis of a 1 lb. liver containing 20 % oil from data given by Lovern *et al.* [1933].

To study the distribution of fat and vitamin A in the tissues two specimens were used (length 5 ft. 1 in., 5 ft. 1½ in.; weight 51 lb. 7½ oz., 53 lb. 1½ oz., respectively) taken at 150 fathoms on 2. vii. 35. The liver oils were prepared in the usual way, but the remaining tissues were steam-cooked and then pressed to remove surplus water prior to continuous extraction with light petroleum (B.P. 50–70°).

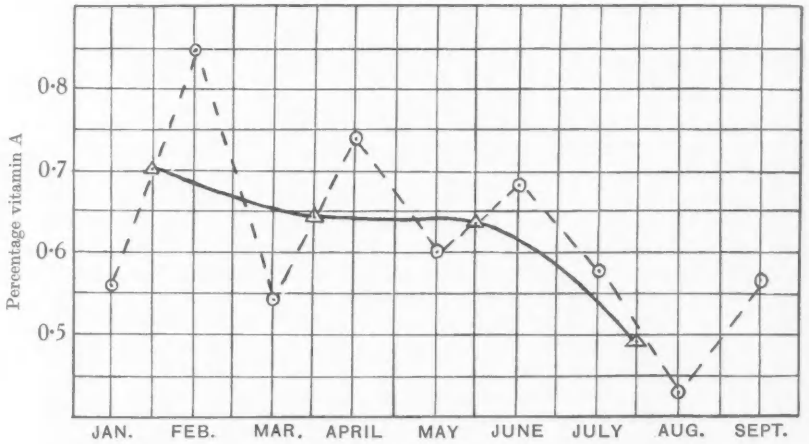


Fig. 1. Seasonal variations in the vitamin A content of Cook Strait ling liver oil.
⊙— — ⊙ Mean monthly values. △——△ Mean bi-monthly values.

Table II. *Distribution of vitamin A in the tissues of the ling*

| | % wt. of total fish | Oil content as % total oil in fish | Oil content of tissues % | Carr-Price value | % vitamin A | % vitamin A of total vitamin A in fish |
|---|---|---|---|---|---|---|
| Liver, | 8·3 | 98·9 | *ca.* 35 | 410 | 0·53 | *ca.* 97·9 |
| Viscera, (excluding liver) | 5·1 | 1·0 | 0·6 | ·820 | 1·05 | 2·1 |
| Body, | 67·2 | 0·1 | 0·04 | No test | — | — |
| Head, | 19·4 | Trace | 0·004 | No test | — | — |

The higher values of 0·11 and 0·18 % for the fat content of two specimens of ling flesh examined by Johnson [1921] were probably partly due to more efficient extraction by means of ether and partly to the fact that the present results have been expressed as a percentage of the whole body including both flesh and bones. In contrast to the viscera of the halibut [Lovern *et al.* 1937] which may contain as much vitamin A as the liver, the viscera of the ling yielded only traces of vitamin A, 97·9 % of the total vitamin being found in the liver.

The low yield, calculated as 2 % of the total oil in the fish, and the negative Carr-Price test of the oil obtained from four typical mature roes (av. wt. 566 g., 0·9 % fat) taken on 28. ix. 35, suggest that spawning has very little effect on the oil and vitamin reserves of the liver.

Table III summarizes the data on the vitamin A potency of some ether-extracted oils prepared from whiptail (*Macruronus novae zelandiae*) which is the chief food of the ling [cf. Phillipps, 1926].

Table III. *Vitamin A content of Cook Strait whiptail*

| | Wt. g. | % oil (ether extract) | Carr-Price value | % vit. A | Total vit. A mg. | Remarks |
|---|---|---|---|---|---|---|
| $W_1$ (whole fish) | 1246 | 0·72 | 75 | 0·10 | 90 | Taken practically undigested from one of the ling described in Table II |
| $W_2$ (body and head) | 1132 | 0·17 | 23·6 | *ca.*0·03 | *ca.* 0·6 | Taken by line from the ling fishing grounds, 23. vii. 35 |
| $W_2$ (viscera excluding liver) | 46 | 1·51 | 280 | 0·36 | 2·7 | |
| $W_2$ (liver) | 8·6 | 15·7 | 370 | 0·47 | 6·3 | |
| $W_3$ (liver) | — | — | 68 | 0·10* | — | — |
| Ling liver | 694 | 35 | 490 | 0·615 | 1494 | — |

* $E^{1\%}_{1\text{ cm.}}$ 328 mμ, 1·6.

## Discussion

The results of previous investigations suggest that diet and changes in oil content are important factors in determining seasonal variations in the vitamin potency of fish oils [cf. Drummond & Hilditch, 1930; Lovern *et al.* 1933].

Changes in the oil content of the liver are frequently connected with spawning. The cod, for example, which has been studied in detail by Drummond & Hilditch [1930], ceases to feed at such times and a considerable proportion of oil is transferred to the gonads [cf. Hjort, 1914], leaving the vitamin reserves practically unimpaired. The residual liver oil, therefore, contains a higher concentration of vitamin A than when the fish is feeding normally. The reverse process may take place during intensive feeding on a diet relatively deficient in vitamin A. Thus the intensive feeding of the groper during October and November is associated with an infiltration of fat into the liver which reduces the vitamin A potency of the oil [Shorland, 1937]. Bills *et al.* [1934] noted that the potency of halibut liver oil was related to the oil content of the liver, but the decrease in vitamin A content from January to August was disproportionately greater than the increase in oil content, suggesting that other factors in addition to dilution controlled the potency of the oil.

In the case of North Sea halibut, the spawning effect is completely masked by pronounced seasonal variations which follow closely the normal rhythm of the diatom cycle, and as the abundance of diatoms is the only available measure of the carotene content of the sea, it is suggested [Lovern *et al.* 1933] that the great abundance of these organisms in the spring may be responsible for the increased potency observed during that period. A similar diatom rhythm has been found in the southern hemisphere [Dakin & Colefax, 1933] but the diatomaceous increase in the spring, so far as the ling is concerned, coincides with the period of minimum potency of the liver oil.

To judge from the time of appearance of mature roes it is probable that spawning takes place towards the end of August, when the potency of the liver oil is at a minimum. The present data suggest that spawning imposes an insignificant drain on the oil and vitamin A reserves of the liver, and, further, that the yield of oil remains constant for the greater part of the season. Observations over the past few years, however, show that during August and September, the cooked livers are not so firm as at other times and are therefore less amenable to pressing. If, therefore, the ling eats little or no food while spawning, and the vitamin A reserves are partially depleted during this period, the reduced potency observed at this season would be expected to occur.

Many fish, such as cod and halibut, appear to store large amounts of vitamin A in their liver although their food is relatively deficient in this vitamin [Drummond & Hilditch, 1930; Lovern *et al.* 1933; Macpherson, 1933], so that liver oils from older fish usually contain higher concentrations of vitamin A. Moreover, within a given species, as both the oil content and relative weight of the liver increase with age, the total vitamin A reserves become disproportionately greater. In the case of the ling, however, the food (cf. Table III) is shown to be relatively rich in vitamin A so that little storage is necessary to produce a liver rich in vitamin. In confirmation of this the larger livers from the older fish have not been found to yield more potent oils than the smaller livers of the younger fish (cf. Table IV). In instances where the total oil content is given, the vitamin A content may be calculated as 246 mg./100 g. tissue for livers of less than 700 g., whereas the heavier livers show 203 mg. The data given by Edisbury *et al.* [1937] for fresh water eel (*Anguilla aucklandi* Rich.) also suggest that age has no marked influence on the vitamin A potency of the oil or tissues of the liver. In contrast to the ling, however, other tissues in the eel form a depot for vitamin A so that if the whole of the vitamin were stored in the liver, the liver oils would indeed show a well-marked progressive increase in potency with age.

Table IV. *Relation between weight of liver and vitamin A content of oil*

| | Wt. of liver | | | | |
|---|---|---|---|---|---|
| | 400–500 g. | 500–600 g. | 600–700 g. | 700–800 g. | Above 800 g. |
| No. samples | 4 | 5 | 8 | 6 | 7 |
| % vitamin A (mean value) | 0·77 | 0·67 | 0·52 | 0·64 | 0·52 |

Accepting an average weight of 20 lb. for ling [*New Zealand Marine Dep. Rep.* 1933] the liver on the average comprises 7·5 % of the headed and gutted fish as compared with 12·4 % in the case of the larger specimens described in Table II. This increased proportion of liver in the larger fish has been generally established for most other species examined. Therefore it is probable that despite the slightly lowered potency of oil from the larger livers as compared with the smaller, the total vitamin A reserves increase proportionately with age, but the extent of the increase is very much less marked than in either cod or halibut.

## Summary

The vitamin A content of ling (*Genypterus blacodes*) liver oil slowly decreases from a maximum value of 0·7 % during January–February to a minimum value of 0·5 % during July–August. Changes in the vitamin potency of the oil are apparently related to spawning, when the vitamin reserves are used up to a much greater extent than the oil itself. In contrast to the fish hitherto studied by other workers the vitamin A content of ling liver oil does not increase with age, though, owing to the greater relative proportion of liver in the older fish, it is probable that there is a slight proportionate increase in the vitamin A content of the whole fish.

The author wishes to thank the directors of the British Drug Houses, Ltd., London, for generously placing their analytical laboratories at his disposal. Thanks are also due to Dr R. A. Morton of Liverpool in whose laboratories several spectrographic assays were made, to Prof. T. P. Hilditch for his continued interest and encouragement and to the Department of Scientific and Industrial Research, New Zealand, for a scholarship.

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# LXV. THE SPECIFICITY OF XANTHINE OXIDASE

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A DETAILED study of the question of identity of xanthine oxidase and the Schardinger enzyme was made in 1935 [Booth]. Most of the existing evidence in favour was confirmed while that against was shown either to have another explanation or to be insufficiently controlled. In addition several new lines of evidence were presented. The results of the investigation pointed strongly to identity though the hypothesis could not be regarded as rigidly proved. Since then other authors [Basu & Mukherjee, 1936; Dixon & Keilin, 1936; Lemberg *et al.* 1936; Michlin *et al.* 1936] have extended certain of the earlier evidence, each concluding in favour of identity. There remain however certain points in the literature against this view. An investigation of most of these is reported in this paper and yields no support for the separate enzyme theory. Considering the mass of evidence from the work of several authors there can be no doubt that xanthine oxidase activates aldehydes. Nothing short of preparation of each enzyme completely free of the other can be taken as proof of non-identity. The apparent suppression of one enzyme by an inhibitor is insufficient.

Because purines and aldehydes are both activated by the same enzyme, xanthine oxidase offers a case of dual specificity of unusual interest on two counts: firstly because all aldehydes hitherto tested are activated, while as Coombs [1927] showed, only 5 of some 17 purines which he and Morgan *et al.* [1922] tested are activated; and secondly on account of the chemical difference between purines and aldehydes. This specificity has been examined in detail in the present paper which includes: (*a*) an extension of the list of purines activated, with relative oxidation rates; (*b*) rates of oxidation of over 30 aldehydes; (*c*) a proof that cozymase is not activated; (*d*) particulars of experiments with substances related to aldehydes; (*e*) suggestions for the use of xanthine oxidase as a biochemical reagent for detecting aldehyde groups.

## *Methods*

The Dixon & Kodama [1926] "whey" preparation of xanthine oxidase was made from Grade A milk. The *p*H was that of the 0·25 *M* phosphate buffer, 7·2, in which the enzyme was dissolved, except that, when concentrated enzyme solutions were used the *p*H was lower on account of the buffering effect of the ammonium sulphate present in the preparation. A trace of octyl alcohol was added for prolonged experiments.

The "cozymase" preparation was made from yeast by the method of Myrback & Örtenblad [1935] and taken as far as the phosphotungstic stage. The last process was repeated and the resulting solution concentrated by low pressure distillation.

The anaerobic experiments were carried out in specially designed Keilin-Thunberg vacuum tubes. Unless stated differently, each tube contained 3 ml.

[1] George Henry Lewes student.

buffered enzyme solution, 1 ml. 0·0005 *M* methylene blue, *x* ml. substrate and 1–*x* ml. water or another solution. Substrates commonly used were 0·2 ml. 0·007 *M* hypoxanthine and 0·8 ml. 0·02 *M* salicylaldehyde. The tubes were evacuated and washed out 3 times with nitrogen. In general the substrate was placed in the hollow stoppers and tipped into the enzyme mixture after 3 min. equilibration at 38°. The anaerobic technique is rapid and convenient, but has certain limitations. The accuracy is probably greatest for reduction times of between 5 and 10 min. and these were obtained where possible. Even then the reduction times are liable to an error of ± 5 %. In some experiments 80 % reduction was taken as end point but the discrepancy between duplicates was greater than when complete reduction was taken as end point, on account of difficulty in matching the colours. The reduction velocity at the end was not markedly less than the mean. The times for complete reduction were therefore recorded.

The aerobic experiments were done in Warburg manometers, the substrate being tipped in from a side bulb after 15 min. equilibration. The purines were dissolved in alkali which may absorb $CO_2$ during manipulations. On tipping into the buffer after equilibration some of the $CO_2$ is liberated. Hence for total oxygen uptake with purines alkali was placed in the pots, although it must be avoided in the case of aldehyde oxidation. The temperature of the thermostat bath in all cases was 38°.

### Activators and accelerators

*Effect of fat.* Dixon & Thurlow [1924] found that boiled cream accelerated the reduction of methylene blue in presence of hypoxanthine but not in presence of acetaldehyde. Although they made no such claim this result might be quoted in favour of non-identity of the enzymes. Wieland & Rosenfeld [1930] found that a colloidal suspension of cholesterol had an accelerating effect on xanthine oxidase.

In order to study this effect an enzyme preparation was dried in a vacuum desiccator, defatted by several extractions with ether, ground finely, again dried and extracted three times with washed, dried ether. The acceleration, by boiled cream, of methylene blue reduction with hypoxanthine was confirmed. But an acceleration with salicylaldehyde as substrate was also observed. The grease used for sealing the vacuum tubes had a similar effect. Hence the joke about the tap grease complicating the results has some foundation! Greaseless rubber-stoppered vacuum tubes were therefore used, and substrates added from cupsticks. Accelerations were observed with olive, linseed and paraffin oils, saponin and taurocholate. In all cases, including that of the boiled cream, the accelerations were under 20 % and variable, being greater when the tube was shaken vigorously. Shaking aids emulsification and the result lends support to Dixon & Thurlow's theory that the effect is due to "the catalytic action of the large surface presented by the fat". Apart from this no complete explanation has yet been found, but one conclusion, germane to the present investigation, is clear, namely that fat has the same effect on both enzymes. Dixon & Thurlow's lack of effect with acetaldehyde can be explained as follows. The substrate concentration curve for acetaldehyde only reaches a maximum at high concentration. Although no figures are given, if we may judge from other experiments in the same paper, these authors used a concentration which was on the rising part of the curve. Acetaldehyde is soluble in most fats and oils; part of it was therefore removed from the aqueous enzyme phase by the emulsified fat. The lowering of the concentration of aldehyde in the aqueous phase annulled the effect of the fat. This explanation receives support from my experiments with salicylaldehyde. Boiled cream slightly accelerated the reduction time when the substrate was

present in optimal concentration (0·003 *M*) but retarded it when present in suboptimal concentration (0·002 *M*).

Boiled cream also accelerated the aerobic oxidation of hypoxanthine but the effect was barely outside experimental error—6 %.

*Increase in activity of milk on standing.* Wieland & Macrae [1930] found that when milk stands (with preservative) the xanthine oxidase, but not the Schardinger enzyme, activity increases and reaches a constant value on the third day. This I have been unable to repeat. Toluene and octyl alcohol were respectively added to two samples of milk which were stored at 2° and tested daily with hypoxanthine and salicylaldehyde. After 1 day methylene blue was reduced 5 % faster in each case but the increased activity was accounted for by development of a small "blank". On the third day both activities declined. Controls showed that the enzymes were not affected by the antiseptics.

*Rennin.* Dixon & Kodama [1926] found that their whey preparation had over 3 times the enzyme activity which could be accounted for by the activity of the original milk. They suggested that the rennin used in preparing the enzyme had liberated xanthine oxidase from an inactive precursor. A similar increase in Schardinger enzyme activity would provide evidence for identity. Unfortunately neither I nor the original authors have been able to repeat the observation. Milk does not contain an inhibitor which is removed during the preparation of the enzyme, for the activities of milk and enzyme preparation are exactly additive.

## Inhibitors

*Substances related to substrates.* Prof. H. O. L. Fischer kindly sent me some 6-amino-8-hydroxypurine. This purine inhibited both hypoxanthine and aldehyde oxidation considerably. The reduction times of methylene blue with hypoxanthine were: without aminopurine 8 min.; with 0·0003 *M* purine 25 min. With formaldehyde in optimal concentration the times were 8 and 130 min. respectively.

Several substances suspected as possible inhibitors of one enzyme system were found to inhibit neither. Again the results with both enzymes ran parallel. Among these substances were: 7-methylhypoxanthine, allantoin, pig serum, chloral hydrate, butylchloral hydrate, glucose, gluconate, propionate, octyl alcohol and trithioformaldehyde.

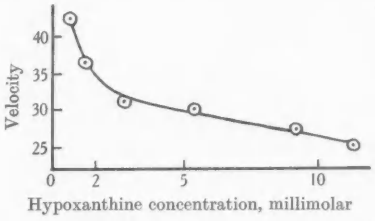


Fig. 1. Hypoxanthine concentration curve. Warburg manometric method. 38°. 2 ml. buffered enzyme solution *p*H 7·2 + 0·1 ml. catalase solution + 0·3 ml. hypoxanthine solution. The velocity is expressed as $\mu$l. oxygen per hour per 2·4 ml. solution.

Originally one of the strongest pieces of evidence for identity was the inhibition of the Schardinger enzyme by uric acid anaerobically. Anaerobically hypoxanthine inhibits xanthine oxidase strongly, and the fall in the curve in Fig. 1 suggests that aerobically it also inhibits slightly. On the other hand

neither uric acid nor guanine inhibit during the aerobic oxidation of hypoxanthine, as shown by Dixon & Keilin [1936] using the caseinogen enzyme preparation. Similarly, using the whey preparation I have found that neither 0·0036 nor 0·001 *M* uric acid had any effect on the oxygen uptake rate with 0·0017 *M* hypoxanthine, even when added before the substrate. Uric acid does however inhibit the oxygen uptake rate with furfuraldehyde [Booth, 1935]. The fact that xanthine oxidase is unaffected aerobically by its most typical anaerobic inhibitor emphasizes the need for care in interpreting results obtained with inhibitors.

## Nomenclature

The suggestion is offered that the enzyme be called xanthine oxidase even when its aldehyde activity is being discussed. The name Schardinger enzyme is superfluous. The eventual classification of oxidases and dehydrogenases may require a renaming of xanthine oxidase but one name should still suffice.

## Purines and xanthine oxidase

Dixon [1926] tested a large assortment of possible substrates with a xanthine oxidase preparation and found that, outside purines and aldehydes, none were activated. Coombs [1927] tested several purines with a whey preparation and divided them into groups:

(1) Activated and adsorbed;
(2) Adsorbed but not activated;
(3) Neither adsorbed nor activated.

His criterion for "adsorption" was inhibition of the enzyme when hypoxanthine was used as substrate for reduction of methylene blue. In the first group were placed only hypoxanthine, xanthine, 6:8-dihydroxypurine, 2-thioxanthine and adenine. In the second group were guanine, certain methylated purines and a dimethylated guanine. In the third group were other dimethylated purines. He concluded that adsorption and activation were dependent upon the completeness of the purine ring, that activation was prevented and adsorption lessened by methyl groups and that adsorption was increased by amino groups. Examination of the formulae shows that (*a*) methylation at 2, 6 or 8 positions would lessen (and in most cases prevent) oxidation, (*b*) *any* substituent (including methyl) in almost any other position, except possibly 7, would prevent enolization and (*c*) an amino group at 2 or 6 might aid enolization. Enolization may be a necessary condition for activation. It would be interesting to test deoxyxanthine, and purines with substituents other than methyl groups, particularly amino, in positions preventing enolization.

Prof. H. O. L. Fischer has kindly given me samples of four purines related to hypoxanthine. These are from the collection of purines prepared by Emil Fischer. The following three reduced methylene blue in presence of the active (but not in presence of boiled) enzyme

| | Velocity |
|---|---|
| 8-hydroxypurine | 6 |
| 6-amino-2-hydroxypurine | 3·5 |
| 6-amino-8-hydroxypurine | 6 |

with the comparative velocities shown, the rate with hypoxanthine being taken as 100. That the reduction was not due merely to traces of impurities was shown by determining the total oxygen uptake. The 6-amino-8-hydroxypurine took up

70$\mu$l. oxygen per mg. (mean of several runs with different initial substrate concentrations). The theoretical value for one atom of oxygen per molecule is 76$\mu$l. The rate of uptake with 6-amino-2-hydroxypurine in presence of a strong enzyme solution fell off gradually. After 8 hr. the oxidation was still proceeding slowly and a little under one atom of oxygen per molecule had been taken up. The course of the oxygen uptake with 8-hydroxypurine, determined at a series of initial concentrations (Fig. 2), is in two distinct stages. After the uptake of

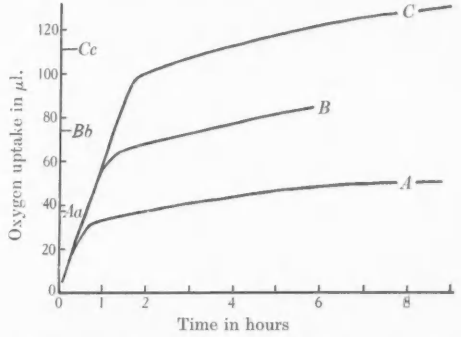


Fig. 2. Oxygen uptake with 8-hydroxypurine in presence of xanthine oxidase (whey preparation) plus catalase. Warburg manometric method. 38°. *A*, with 0·44 mg. purine; *B*, 0·88 mg.; *C*, 1·3 mg. *Aa*, *Bb*, *Cc*, theoretical values for one atom of oxygen per molecule for *A*, *B*, *C*, respectively.

approximately one atom per molecule the rate fell to one-tenth of the initial rate. Presumably the first product is 2:8-dihydroxypurine which may be only very slowly oxidized; the alternative product—6:8-dihydroxypurine—is improbable since that is known to be oxidized as rapidly as xanthine [Coombs, 1927]. The suggestion is made that 2:8-dihydroxypurine may therefore be added to the list of purines activated by xanthine oxidase. These results, together with the initial relative oxygen uptake rates, are brought together in Table I. Hypoxanthine and adenine were also used with the same enzyme

Table I. *Oxygen uptake with purines*

| Substrate | Relative initial rate | Atoms oxygen per molecule | Benedict's uric acid test |
|---|---|---|---|
| Hypoxanthine | 100 | 2 | + + |
| Adenine | 6 | 2 | + |
| 8-Hydroxypurine | 6 | 1* | + |
| 2:8-Dihydroxypurine | 0·6 | | |
| 6-Amino-8-hydroxypurine | 7 | 1 | + |
| 6-Amino-2-hydroxypurine | 4·5 | Nearly 1 | + |

* The rate then fell off to one-tenth.

preparation and are included for comparison. In all cases the observations were discontinued within 9 hr. to avoid bacterial growth. The falling off in oxygen uptake was not due to destruction of the enzyme. This was demonstrated by methylene blue reduction experiments using the final contents of the Warburg pots as enzyme solution. Benedict's uric acid test was in each case positive with the oxidation product though not with the original substance. The test is, however, unspecific.

To test whether deamination by another enzyme precedes oxidation the two aminopurines were incubated anaerobically with the enzyme preparation in Thunberg tubes for various periods before tipping in methylene blue from the hollow stopper. In the case of the 6-amino-2-hydroxypurine a half-hour incubation had no effect but a 2 hr. incubation shortened the reduction time slightly. In the case of the 6-amino-8-hydroxypurine the reduction time was increased slightly by previous incubation. The results by this method being inconclusive ammonia was determined after incubation of each purine with the enzyme preparation. The latter was made by saturating whey with magnesium sulphate instead of half-saturating with ammonium sulphate (to minimize the initial ammonia content), drying the precipitate, defatting with ether, dissolving in phosphate buffer in the ordinary way and centrifuging. In presence of a 10% solution of this preparation the two aminopurines and adenine each reduced methylene blue in a few minutes, proving that the preparation was enzymically very active. These three purines were incubated anaerobically each with aliquot portions of this enzyme solution. After 12 hr. the ammonia concentration, determined by the method of Parnas, was identical in all cases with that of the blank without added substrate. Similarly no ammonia was produced from adenine by 12 hr. aerobic incubation with the enzyme preparation. These purines are therefore assumed to be directly activated by xanthine oxidase. The non-production of ammonia from adenine confirms Dixon & Lemberg's [1934] finding that methylene blue was reduced by adenine even in presence of purified xanthine oxidase.

To complete the series there still remain to be tested one more probable substrate, namely 2-hydroxypurine, two more possible substrates, namely purine and deoxyxanthine, and the various thiopurines.

The fourth purine (from H.O.L.F.) was 7-methylhypoxanthine. It neither reduced methylene blue nor inhibited the reduction with hypoxanthine or aldehyde.

### Cozymase and xanthine oxidase

Dixon & Lemberg [1934] showed that the reduction of methylene blue by certain nucleosides in presence of the undefatted whey preparation was really due to hypoxanthine which had been split off by nucleosidases. When the xanthine oxidase preparations were freed from these hydrolysing enzymes the reduction no longer occurred. A preparation of "cozymase" similarly reduced methylene blue in presence of crude—though not in presence of purified—xanthine oxidase, but these authors did not determine whether cozymase itself or some other substance in the preparation was concerned here or whether cozymase was functionally inactivated. Reports that xanthine oxidase inactivates cozymase have since appeared. Lipmann [1935] found that a xanthine oxidase preparation inactivated cozymase aerobically but not anaerobically. Andersson [1936] reported that the Schardinger enzyme inactivated cozymase both as coenzyme of alcoholic fermentation and as coenzyme of dehydrogenation. Reinvestigation has produced the following results.

"Cozymase" was incubated anaerobically with a strong xanthine oxidase preparation. After 15 min. the mixture was boiled and tested. The solution failed as coenzyme for the lactic acid dehydrogenase system prepared from pig heart by the method of Green & Brosteaux [1936]. It also failed to promote fermentation by dialysed yeast juice. The eight necessary controls were done (e.g. cozymase was found to be functionally active after being boiled with boiled xanthine oxidase preparation) and the experiments were repeated with other

preparations. There cannot be any suitable hydrogen acceptor in either the enzyme or "cozymase" preparations for no uric acid is produced by incubation of the enzyme with hypoxanthine or "cozymase", and oxygen is excluded. Hence the process is not oxidative and the cozymase-inactivating enzyme is evidently a nucleosidase.

The xanthine oxidase preparation (with its contained nucleosidases) can be a useful reagent in coenzyme studies. Certain systems, for instance α-glycerophosphate, succinic and other dehydrogenases appear not to require a coenzyme though that may be only on account of separation difficulties. If such dehydrogenase is not inhibited by the xanthine oxidase preparation and does not itself behave as a substrate for the reduction of methylene blue in presence of the latter, it is unlikely that a coenzyme of the nucleoside type is concerned.

Harrison [1933] showed that xanthine oxidase (milk preparation) reacted with glucose dehydrogenase, except when the latter was protected by bisulphite, and concluded that the activating group on the glucose enzyme is an aldehyde. This result now loses some of its significance, for the coenzyme would be inactivated by a nucleosidase in the milk preparation.

### Aldehydes activated by xanthine oxidase

The original substrate used by Schardinger [1902] was formaldehyde. Since that time other aldehydes have been used, particularly acetaldehyde, and now it is generally assumed, though not proved, that all aldehydes are activated by the one enzyme. The specificity towards aldehydes has therefore been examined in detail.

The aldehydes in Table II reduced methylene blue in presence of the enzyme preparation from milk. At least all of those marked † have been used by other workers. Those marked * have also been shown to take up oxygen in presence of the enzyme. Comparative reduction rates (taking the hypoxanthine rate as 10,000) are given, but these must be accepted as only approximate for various reasons, some of which are as follows. (*a*) Certain aldehydes, e.g. formaldehyde, *o*-phthalaldehyde, destroy the enzyme. (*b*) Most aldehydes are only slightly water-soluble: with these the rates might be dependent on the degree of emulsification, amount of shaking and other factors. (*c*) As the substrate concentration curves are in general not flat several concentrations should be used and the maximum rate recorded: the experimental technique however is then unjustifiably complicated except with the reasonably soluble aldehydes. Curves were obtained for 7 of the more soluble, which are indicated by ‡. In all other cases a drop of the liquid or 2 mg. of the solid was placed in a Thunberg tube stopper and vigorously shaken with the enzyme-dye mixture at the end of the usual 3 min. incubation. The aldehydes were used as fresh as possible; liquids of doubtful purity were redistilled. Because the range of velocities is great the enzyme and methylene blue concentrations were varied considerably. In most cases a control was performed with boiled enzyme: no reduction in 24 hr. is indicated by 0 and a slow reduction time by S; when the reduction was comparable with that for the unboiled enzyme the ratio of the two rates is shown. In the last case a test was made with protein-free buffer: if the methylene blue was reduced at all this is indicated by B. The *p*H of the solutions was always below 7·3. Propaldehyde and *iso*butaldehyde were also activated but the reduction rates were not compared with a standard. A test could not be made with *o*-phthalaldehyde as it reacted with the protein of the enzyme preparation, producing a black substance. Methylglyoxal and *n*-valeraldehyde have been shown by Dixon & Lutwak-Mann [1937] to be activated by xanthine oxidase.

Table II. *Aldehydes activated by xanthine oxidase*

| Aldehyde | Methylene blue reduction rate | Boiled enzyme |
|---|---|---|
| Acetaldehyde†*‡ | 7250 | S |
| Aldol | 700 | |
| Anisaldehyde | 2800 | 0 |
| Benzaldehyde | 8000 | 0 |
| *n*-Butaldehyde | 60 | 0 |
| Cinnamaldehyde | 4100 | 0 |
| Crotonaldehyde | 70 | S |
| Cuminaldehyde | 580 | 0·3 B |
| Decanaldehyde | 80 | 0 |
| Duodecanaldehyde | 20 | 0 |
| Dimethyl-*p*-aminobenzaldehyde† | 40 | |
| Formaldehyde†*‡ | 9000 | 0 |
| Furfuraldehyde*‡ | 7500 | 0 |
| Glyceraldehyde‡ | 140 | 0·5 |
| Heptanaldehyde | 240 | S |
| *o*-Hydroxybenzaldehyde (salicylaldehyde)*†‡ | 6000 | 0 |
| *m*-Hydroxybenzaldehyde | 8000 | 0 + |
| *p*-Hydroxybenzaldehyde | 13000 | 0 |
| *β*-Indolealdehyde | 140 | 0 |
| *o*-Methylbenzaldehyde | 1500 | 0 |
| *o*-Methoxybenzaldehyde | 210 | 0 |
| *o*-Nitrobenzaldehyde | 20 | 0 |
| Octylaldehyde | 2 | 0 |
| *p*-Phthalaldehyde | 130 | 0 |
| Phenylacetaldehyde | 80 | 0·7 |
| Phenylglyoxal | 20 | S |
| Phenylpropaldehyde | 240 | 0·2 B |
| Piperonal†*‡ | 5600 | 0 |
| Protocatechuicaldehyde | 100 | |
| *iso*Valeraldehyde | 5 | 0 |
| Vanillin†*‡ | 1050 | 0 |

The reduction rates of methylene blue were additive with formaldehyde and piperonal in suboptimal concentrations and competitive in optimal concentrations. This confirms the accepted view that only one enzyme is concerned in the activation of these two aldehydes. But as formaldehyde (the first member of the series) differs more from piperonal than the rest differ from each other it may be taken that all are activated by the same enzyme. Further, since these are representative of such widely differing chemical types it seems probable that all aldehydes would be activated. In contrast to the effect on purines, methylation does not prevent activation of aldehydes. Evidently xanthine oxidase could be used, provided that suitable controls were performed, as a biochemical reagent for testing for the presence of an aldehyde group.

### Substances related to aldehydes

A number of substances related to aldehydes has been tested: chloral hydrate, butylchloral hydrate, paraldehyde, glucosone and mannose. Although a very strong xanthine oxidase solution was used no reduction of methylene blue was observed in 10 hr. Neither chloral hydrate, butylchloral hydrate, glucose nor gluconate affected the reduction rates using hypoxanthine or salicylaldehyde, even after previous incubation with the enzyme; hence these substances do not combine with the enzyme.

SUMMARY

1. Evidence against the identity of xanthine oxidase and the Schardinger enzyme is investigated but not substantiated. The conclusion is reached that xanthine oxidase has now been proved identical with the Schardinger enzyme, and the suggestion is offered that the latter name be discontinued.

2. Four purines have been added to the existing list of 5 activated by xanthine oxidase and the rates of their oxidation determined: 8-hydroxypurine, 6-amino-2-hydroxypurine, 6-amino-8-hydroxypurine and 2:8-dihydroxypurine. The direct activation of the two aminopurines has been proved, and that of adenine has been confirmed, by demonstrating that previous deamination does not occur.

3. Cozymase is destroyed by a nucleosidase but not by xanthine oxidase.

4. A list of 35 aldehydes activated has been compiled.

5. Substances related to aldehydes—mannose, glucosone, gluconate, chloral hydrate, butylchloral hydrate, paraldehyde—do not combine with the enzyme.

6. It is concluded that in addition to purines all true aldehydes—but only true aldehydes—are activated.

I should like to thank Drs M. Dixon, D. E. Green and H. A. Krebs for criticism and advice and the last for the hospitality of his laboratory for certain of the manometric experiments, Prof. E. Friedmann for the sample of methylbenzaldehyde, Dr D. D. Woods for the indolealdehyde, and Mr K. Harrison for the glucosone.

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# LXVI. THE XANTHINE OXIDASE-ALDEHYDE SYSTEM

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Booth [1938] concluded that xanthine oxidase and the Schardinger enzyme are identical and that the one enzyme activates all aldehydes. Although xanthine oxidase has been much studied as to its purine activity, less is known about its aldehyde activity. In this paper certain aspects of the dynamics of the enzyme-aldehyde system will be considered especially in regard to differences from the enzyme-purine system. The exceptional dual specificity of xanthine oxidase offers an experimental example of the fact that many properties of enzyme systems belong not to the enzymes but to the chemical reactions catalysed.

The enzyme preparation was made from whey by the method of Dixon & Kodama [1926] and dissolved in phosphate buffer *p*H 7·2 as required. Other experimental details are described by Booth [1938].

### *Destruction of enzyme by substrate*

While there is no experimental indication that purines destroy the enzyme, formaldehyde destroys it anaerobically as the following experiment shows. A series of Thunberg tubes was set up with the same amount of methylene blue and buffered enzyme solution in each and with formaldehyde in optimum concentration as determined in Fig. 1. In the 1st tube the aldehyde was placed in the stopper with the methylene blue. In the 2nd tube the aldehyde was placed in the stopper but the methylene blue was put in the tube with the enzyme. In the other tubes the methylene blue was placed in the stoppers and the aldehyde with the enzyme. The tubes were incubated together, the first three being tipped after the usual 3 min. in the bath (+10 min. at room temp. necessary for greasing, evacuating etc.), and the others at graded intervals. The results in Table I show that the reduction times of the first two were the same but that

Table I. *Destruction of enzyme by aldehydes*

| No. | In tube | In stopper | Tipped after min. | Reduction times: Formaldehyde min. | Reduction times: Acetaldehyde min. |
|---|---|---|---|---|---|
| 1 | Enzyme alone | Aldehyde + methylene blue | 3 | 7·9 | 8 |
| 2 | Enzyme + methylene blue | Aldehyde | 3 | 7·9 | 8 |
| 3 | Enzyme + aldehyde | Methylene blue | 3 | 140 | 9 |
| 4 | ,, | ,, | 8 | 300 | 10 |
| 5 | ,, | ,, | 12 | Incomplete | 10 |
| 6 | ,, | ,, | 60 | Partial | 11 |

the times were increased by incubating the enzyme with the aldehyde. The last tubes were not completely reduced even overnight. Evidently formaldehyde destroys the enzyme sufficiently rapidly to make it an unsuitable substrate for quantitative studies, and presumably does so by reacting with amino groups on

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the enzyme molecule: these do not necessarily constitute the "active group" of the enzyme since no appreciable protection was afforded by uric acid. The experiment was repeated using acetaldehyde in concentration slightly above optimal. Destruction of enzyme by acetaldehyde was negligible in comparison with that by formaldehyde and may have been due to traces of peroxide. There was no indication of destruction by salicylaldehyde, a commonly used substrate. In presence of the enzyme preparation *o*-phthalaldehyde produced a black substance too rapidly to allow its use for methylene blue studies.

### *Michaelis constants*

The optimal concentration of purine varies with the enzyme strength and the method of preparation (i.e. whether whole milk, "casein" or "whey" preparations are used). Hence the "Michaelis constant" is not strictly constant. Variation, however, is not great, and with the enzyme strength ordinarily used the value for hypoxanthine is 0·00015 *M*, i.e. the enzyme has a high affinity for hypoxanthine. The affinity for aldehydes is lower, for instance:

| | |
|---|---|
| Acetaldehyde | 0·062 *M* |
| Formaldehyde | 0·0085 |
| Salicylaldehyde | 0·0014 |
| Furfuraldehyde | 0·004 |

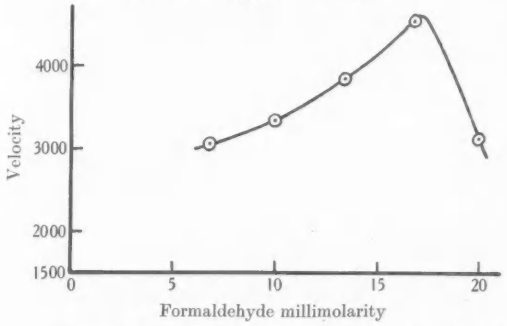


Fig. 1. Substrate concentration curve with formaldehyde. Anaerobic. Velocity is reciprocal of methylene blue reduction times in min. multiplied by 10,000.

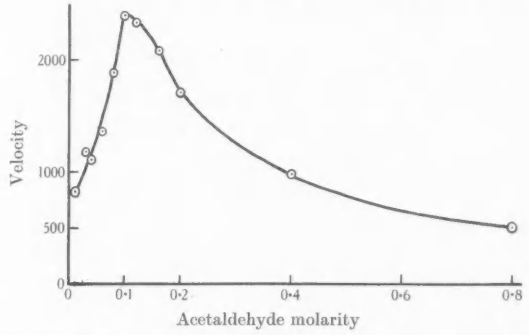


Fig. 2. Substrate concentration curve with acetaldehyde. Anaerobic.

The values for hypoxanthine and salicylaldehyde are taken from the data of Booth [1935], and the values for acetaldehyde and formaldehyde from Figs. 1 and 2. In the case of formaldehyde this estimate of the constant is low since the aldehyde destroys the enzyme and the true optimal concentration is unknown. Acetaldehyde (or an associated impurity) only destroys the enzyme slowly: the optimal concentration shown on the curve is therefore approximately correct. Many aldehydes are only slightly water-soluble. In many cases (e.g. glyceraldehyde) maximum velocity is probably not reached even with saturated solutions and the Michaelis constant cannot be determined.

*Reversibility*

Green [1934] made a study of xanthine oxidase equilibria with purines. He showed that the hypoxanthine-xanthine-uric acid system is reversible and that the potentials obtained agree with those predicted for a 2-stage oxidation. It seemed reasonable to expect that the aldehyde system might also be reversible, but thus far all attempts to reverse the system have failed. The experiments have been of two main types, with many variations.

The first was the colorimetric method of Green [1934]. Benzylviologen was reduced by aldehyde and enzyme in a Thunberg tube; the supposed oxidant was then tipped in from the hollow stopper, reoxidation of the dye indicating reversibility. The neutralized sodium salts of various organic acids have been used as oxidants. Benzylviologen is only partially reduced by the aldehyde system. This limitation was combated in two ways: (*a*) the *r*H of the dye was varied by alteration of *p*H; (*b*) in some experiments the indicator was reduced with a very small amount of hypoxanthine. Reoxidation was never observed.

In the second method hypoxanthine and a neutral solution of the salt of an organic acid were anaerobically incubated with enzyme in a series of tubes. At intervals a tube was opened and tests made for uric acid. If the system were reversible and the acid could be reduced to aldehyde some uric acid should be formed, but no trace was found in many experiments in which the *p*H and the concentrations of the reagents were varied.

The failure to reverse the aldehyde system suggests that the corresponding organic acid may not be the true oxidant. It is noteworthy that propionate does not inhibit the enzyme.

*Oxidation products*

The product of the enzymic oxidation of aldehyde is usually assumed to be the acid but this has never been isolated. Even the theoretical $O_2$ uptake [Keilin & Hartree, 1936] does not irrefutably establish that the acid is directly produced. Enzymically the aerobic oxidation almost certainly occurs in two stages, the first being the production of a powerful oxidizing agent. While this agent may be $H_2O_2$ the following facts support other possibilities.

(1) This agent destroys the enzyme much more rapidly than the oxidizing agent produced by the aerobic oxidation of hypoxanthine. The latter is probably $H_2O_2$ according to Dixon [1925] and Wieland & Rosenfeld [1930]. The difference is not due to protection by purine, for even when uric acid is added to the actively oxidizing aldehyde system the destruction still proceeds faster than in the case of the purine system.

(2) The protection afforded by catalase is variable and only obtained with powerful preparations.

(3) This oxidizing agent performs a coupled oxidation which $H_2O_2$ itself only performs when in much higher concentration. Enzyme, uric acid, and

salicylaldehyde were incubated anaerobically until the uric acid was mostly reduced to hypoxanthine by mixed dismutation [Booth, 1935]. The solution was then aerated at 38°. It was argued that aeration should reoxidize the hypoxanthine to uric acid. However, no uric acid was detectable and such traces as were present before aeration had also disappeared. Furthermore, in presence of aldehyde and the enzyme, uric acid disappeared more rapidly aerobically than anaerobically and no hypoxanthine could be isolated. The uric acid was oxidized, allantoin being apparently one product, since Ro's [1931–32] colour reaction for allantoin was positive and accounted for about one-third of the uric acid. Allantoin was not further oxidized when substituted for uric acid in the above experiment. The coupled oxidation only occurred in presence of the enzyme, aldehyde and $O_2$. That is to say xanthine oxidase and aldehyde together simulate the effect of a weak uricase. $H_2O_2$ also oxidizes uric acid but only when in high concentration. There is no reason to believe that uric acid is removed in this way during aerobic oxidation of hypoxanthine.

Keilin & Hartree [1936] suggested that the substance produced during aerobic oxidation of hypoxanthine may be called nascent $H_2O_2$ because it produced a coupled oxidation of alcohol unobtainable with added $H_2O_2$. The oxidizing agent produced during aerobic oxidation of aldehydes however appears to be more powerful than that produced during oxidation of purines. It may be a peracid or other highly oxidized unstable derivative of aldehyde.

Anaerobically the oxidative process must be different, but might occur also in two stages. In each case the problem of the oxidant remains unsolved.

*Dismutation*

Green [1934] showed that, under the influence of the enzyme, xanthine will dismute to form uric acid and hypoxanthine; and that in presence of hypoxanthine some uric acid disappears, presumably to form xanthine. I have confirmed these observations. On the other hand, aldehydes are not dismuted by xanthine oxidase as the following observations show.

A highly active xanthine oxidase solution was incubated anaerobically with 4% acetaldehyde for 9 hr. The solution was then acidified with $H_2PO_4$ and distilled into NaOH. The distillate was tested by the method of Krüger & Tschirch [1929; 1930] with lanthanum nitrate, iodine and ammonia, which produce a blue colour specific for acetate and propionate. None was found. The same experiment was performed with propaldehyde with the same negative result. Controls showed that the aldehydes had not destroyed the enzyme, and that a positive lanthanum test resulted from addition of either acid to the distillation mixture.

If aldehyde dismutes under influence of the enzyme, yielding alcohol and acid, aldehyde ought to react with hypoxanthine to yield alcohol and uric acid. Experimentally no uric acid was found although various aldehydes were tried. Were alcohols produced it is probable—though not necessarily certain—that they would also be activated by the enzyme, but none has been found to be so activated. Nor do alcohols inhibit the enzyme.

Since these experiments were done a paper has appeared by Dixon & Lutwak-Mann [1937] who have made a thorough investigation into the occurrence of mutase. They found too, using entirely different methods, that xanthine oxidase has no aldehyde mutase activity. They have critically examined the earlier literature in which the Schardinger enzyme is stated to catalyse aldehyde dismutation, and have clarified the position by the preparation of mutase completely free from aldehyde oxidase.

Dismutation or oxido-reduction is closely connected with reversibility, for to oxidize one molecule another must be reduced. The findings that neither reversal nor dismutation occur are therefore mutually confirmatory.


## Summary

Certain dynamics of the xanthine oxidase-aldehyde system which contrast with the xanthine oxidase-purine system are described.

(1) Formaldehyde destroys xanthine oxidase.

(2) Aldehydes have low affinity for the enzyme.

(3) The possible nature of the oxidation product is discussed. Aerobically some product is formed which performs an oxidation of uric acid to allantoin.

(4) Xanthine oxidase does not catalyse the reduction of acids or the oxidation of alcohols to aldehydes respectively.

(5) Xanthine oxidase has no aldehyde mutase activity.


My best thanks are due to Prof. D. Keilin and Dr D. E. Green for criticism and advice during the course of this work.

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# LXVII. THE ENZYME SYSTEM OF *BACT. SUBOXYDANS*

## I. VARIATION OF AEROBIC ACTIVITY WITH AGE OF CULTURE

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(*Received 25 January 1938*)

As described in a previous paper [Butlin, 1936], *Bact. suboxydans*, an acetic acid organism characterized by its property of restricted oxidation in aerobic conditions, was found to possess a strongly oxidizing enzymic system when grown on maize wort agar containing 2 % chalk. In contrast, when grown for 3–4 days on maize wort agar without chalk, of initial *p*H 6·5, it appeared to possess only a simple and restricted power of oxidation, washed suspensions of the organism giving with glucose an $O_2$ uptake equivalent approximately to 1 atom O for each mol. substrate, with very little or no evolution of $CO_2$. It was considered probable that the difference in behaviour of the two types of suspension was due to the influence on cell activity of acid gradually formed on the growth medium. This suggested an examination of the influence of the period between inoculation of the medium and removal of the growth from the medium (hereafter called the growth period) on the aerobic enzymic activities of *Bact. suboxydans*. Recent work by Wooldridge *et al.* [1936; 1937] has shown that the aerobic and anaerobic activities of most bacterial dehydrogenases may vary considerably with the growth period. The present investigation shows that *Bact. suboxydans* exhibits similar variation. Moreover, the results obtained throw light on the enzymic structure of the organism.

### Experimental

The culture of *Bact. suboxydans* used in these experiments was supplied by Prof. A. J. Kluyver of Delft. The media employed for growth consisted of (*a*) maize wort agar of *p*H 6·5, (*b*) maize wort agar with 2 % chalk, contained in flat 16 oz. bottles. After inoculation they were incubated at 30°. One or more bottles of each were withdrawn at required intervals and suspensions prepared as previously described [Butlin, 1936]. The suspensions were made up only roughly to the same density, for it had been previously found that dilution within a wide range made no appreciable difference to aerobic activity, e.g. a suspension of dry weight 23 mg./ml. gave almost identical values for $O_2$ uptake and evolution of $CO_2$ with glucose when diluted to three times its volume. The $O_2$ absorption and $CO_2$ evolution by the suspensions with glucose were measured very soon after preparation by means of the Barcroft differential manometer, the usual technique being followed [Dixon, 1934]. $CO_2$ was measured by the direct method. Both flasks, which differed in volume by less than 50 $\mu$l., contained 1 ml. well washed suspension and 1·7 ml. buffer: to the left was added 0·3 ml. water, to the right 0·3 ml. of 0·1 *M* glucose. The latter was contained in a Keilin tube during equilibration, after which it was dislodged and mixed with the other contents.

A temperature of 30° and phosphate buffers of *p*H 6·0 were used throughout. Theoretically 0·3 ml. of 0·1 *M* glucose takes up 334 $\mu$l. oxygen at N.T.P. for each atom O absorbed by the glucose molecule.

*Variation in aerobic enzymic activities of* Bact. suboxydans *grown on maize wort agar containing 2 % chalk*

Figs. 1 and 2 contain the $O_2$ uptake-time and $CO_2$ output-time curves with glucose as substrate given by suspensions of *Bact. suboxydans* grown for variable periods on maize wort agar containing 2 % chalk. These curves show that the

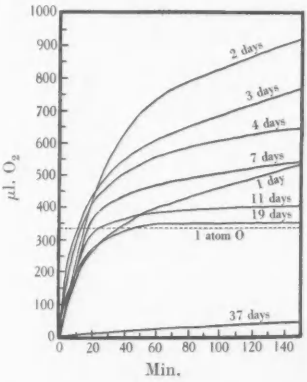


Fig. 1.

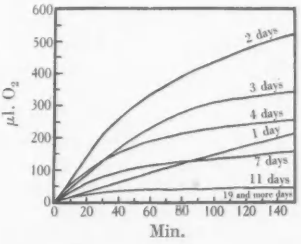


Fig. 2.

Fig. 1. Oxygen uptakes, with 0·3 ml. 0·1 *M* glucose as substrate, of suspensions of *Bact. suboxydans* grown for different periods on maize wort agar containing 2 % chalk.

Fig. 2. Evolution of $CO_2$, with 0·3 ml. 0·1 *M* glucose as substrate, of suspensions of *Bact. suboxydans* grown for different periods on maize wort agar containing 2 % chalk.

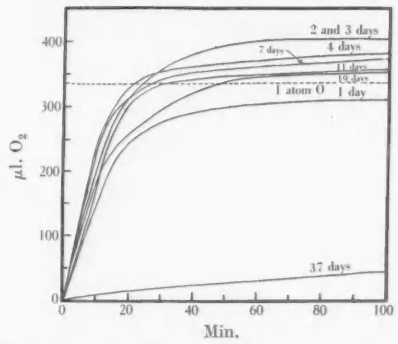


Fig. 3. "$O_2$ uptake unaccompanied by $CO_2$ evolution", with 0·3 ml. 0·1 *M* glucose as substrate, of suspensions of *Bact. suboxydans* grown for different periods on maize wort agar containing 2 % chalk.

activity increases at first, rises to a maximum after 2 days' growth and then gradually diminishes. The behaviour is in general similar to that reported by Wooldridge *et al.* [1936] for most dehydrogenases.

The characteristic feature of the action of all the suspensions with growth periods of 1–7 days is a rapid initial $O_2$ uptake and a relatively slow initial production of $CO_2$. The rate of evolution of $CO_2$ increases and eventually becomes equal to the rate of $O_2$ absorption. This suggests an initial rapid oxidation accompanied by a slower separate reaction in which the evolution of $CO_2$ is equal in volume to the $O_2$ taken up by the glucose.

The 11-day-old culture gives a rapid initial $O_2$ uptake equivalent approximately to 1 atom O per mol. glucose, followed by very slow $O_2$ absorption and very small $CO_2$ evolution. After 19 days' growth there is an $O_2$ absorption approximately equivalent to 1 atom O but no evolution of $CO_2$. There is still slight activity after a growth period of 37 days but none after 7 weeks.

If, as the experiments indicate, there is a definite reaction in which the $O_2$ absorbed is equal to the total $CO_2$ evolved, the difference in volume between total $O_2$ uptake and $CO_2$ output represents an absorption of $O_2$ without an accompanying production of $CO_2$, i.e. some simple oxidation process. This is reflected in the rapid initial $O_2$ uptakes shown in Fig. 1 and is illustrated in Fig. 3, which gives the values for "$O_2$ uptake unaccompanied by $CO_2$ evolution" derived from Figs. 1 and 2. For cultures nineteen and more days old the curves are identical with those for $O_2$ uptake, there being no evolution of $CO_2$. It will be seen that, except for the 37 days' growth period, the curves in Fig. 3 are confined within a narrow range, the final values falling between 330 and 405 $\mu$l. Moreover, apart from the 1-day growth, these final figures show with lengthening period of growth a closer approximation to the theoretical figure for 1 atom O (334 $\mu$l.). It is very probable that these curves represent, up to 334 $\mu$l., the formation of gluconic acid, and beyond 334 $\mu$l. a further partial simple oxidation to 5-ketogluconic acid. These figures of $O_2$ uptake without $CO_2$ evolution remain fairly constant during the growth periods, up to 19 days, in which the total $O_2$ uptake greatly diminishes and the evolution of $CO_2$ ceases altogether. This implies that the enzyme catalysing the simple oxidation to gluconic acid is less susceptible to adverse growth conditions than the enzyme responsible for the reaction involving $CO_2$ production.

It should further be mentioned that considerable acid formation takes place during the experimental growth period. The chalk dispersed throughout the maize wort agar gradually disappears and the growth becomes acid when the accessible chalk has been decomposed.

### *Variations in aerobic activities of* Bact. suboxydans *grown on maize wort agar without chalk*

The results obtained for cultures on maize wort agar without chalk are summarized in Fig. 4, which gives the manometric values after 3 hr. with glucose as substrate for $O_2$ absorption, $CO_2$ evolution and "$O_2$ uptake unaccompanied by $CO_2$ evolution" for the various periods of growth.

The suspension made from a 1-day growth had highly oxidizing activities—characterized by rapid initial $O_2$ uptake, relatively slow initial $CO_2$ evolution, followed by an increase in the latter until its rate of production became equal to the rate of $O_2$ uptake—similar to those of the suspensions obtained from the chalk-containing medium after 1–7 days' growth, and was almost identical in activity with the 3 days' growth from that medium. The 3 days' growth gave very little $CO_2$ evolution and the $O_2$ uptake decreased to a value slightly in excess of the equivalent of 1 atom O. After a growth period of 4 days there was only slow $O_2$ absorption and no $CO_2$ production. Cultures 6 days old gave no sign of aerobic enzymic activity with glucose. This may be contrasted with the

behaviour of suspensions from the chalk medium which still showed slight activity after 37 days' growth.

It is clear too from Fig. 4 that, as with the suspensions from the chalk-containing medium, the value for "$O_2$ uptake unaccompanied by $CO_2$ evolution"

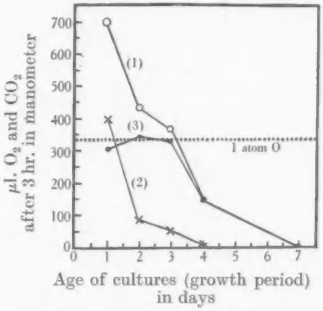


Fig. 4. (1) $O_2$ uptake, (2) $CO_2$ evolution and (3) "$O_2$ uptake unaccompanied by $CO_2$ evolution", after 3 hr. in manometer with 0·3 ml. 0·1 *M* glucose as substrate, of suspensions of *Bact. suboxydans* grown for different periods on maize wort agar without chalk.

remains fairly constant at a level approximately equivalent to 1 atom O, during a period in which the $CO_2$ evolution sinks to zero and the $O_2$ uptake greatly decreases. This again implies the greater resistance to adverse growth conditions of the enzyme catalysing the simple oxidation to gluconic acid.

It was observed that the bacterial growths on this medium became increasingly acid with age and required several washings before testing in the manometer.

## Discussion

In general, variations in the enzymic activity of bacterial cultures depend on the availability of food materials, the nature of the metabolic products and on such factors as temperature, moisture, *p*H and access to $O_2$. In the case under consideration it is at once clear that the chief factor is the acid formed by the organism. The variations have been followed using maize wort agar, with and without chalk, as growth media. The contrast between the growths on the two media is very striking. There is an initial similarity after 1 day's growth, both media giving highly oxidizing suspensions. With increasing growth period there is then a rapid fall in activity of the suspensions from the medium without chalk, until after 6 days aerobic activity is completely lost, whereas suspensions made from the chalk-containing medium show activity even after 37 days' growth. Where acid formed by the organism comes into direct contact with the bacterial cells it is apparent that it has a profound effect on the enzymic activity of the organism. On the chalk-containing medium the acid is neutralized as soon as formed and any decrease in activity of the culture must be due to some or all of the other factors.

It is not surprising that the direct action of acid on bacterial cells should strongly influence enzymic activity. The significance of the experimental data presented in this paper is in their bearing on the enzymic system of *Bact. suboxydans*. Owing to the differential effect of the acid, it is possible to conclude that this system possesses at least two important components. One, less sensitive to acid and more rapid in its catalytic action, is responsible for the oxidation of

glucose probably to gluconic acid and partially to 5-ketogluconic acid, reactions in which no $CO_2$ is evolved. The second component of the enzymic system catalyses the slower action of $O_2$ on glucose which results in an evolution of 1 mol. $CO_2$ per mol. $O_2$ absorbed. This is probably accompanied by the complete breakdown of the glucose molecule, but there is the possibility that the action involves the synthesis of carbohydrates for storage in the bacterial cells [Clifton, 1937]. This second component is much more sensitive than the first to the increasingly adverse conditions, acid and otherwise, which arise as the growth period lengthens. Thus, in the case of suspensions from the chalk maize wort agar the simpler oxidative component persists intact after 19 days' growth, whereas the evolution of $CO_2$ is almost nil after a growth period of 11 days. Similarly, suspensions from the non-chalk medium maintain their simple oxidative enzymic component intact after 3 days' growth, during which period the highly oxidizing factor is rapidly and almost completely inactivated.

It is surprising that *Bact. suboxydans*, which in ordinary liquid culture is characterized by its slight intensity of oxidation, should possess a highly oxidizing factor in its enzymic equipment. A possible explanation of this and further evidence for the enzymic structure outlined above will be presented in a future paper.

## Summary

1. Washed suspensions of *Bact. suboxydans* vary considerably in aerobic enzymic activity according to the period of growth on maize wort agar, with and without chalk.

2. One-day-old cultures on both media give highly oxidizing suspensions, but whereas 37-day-old cultures on the chalk-containing medium still show slight activity, suspensions of 6-day-old cultures from maize wort agar without chalk possess no aerobic enzymic action. This difference is attributed mainly to the formation of acid by the organism and its direct action on the cell if not neutralized.

3. Experimental results suggest that normally *Bact. suboxydans* possesses an enzyme system containing at least two important components. One, less sensitive to acid but more rapid in its action, catalyses a simple oxidation process. The other component catalyses a reaction in which the $O_2$ absorbed is equal to the $CO_2$ evolved.

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# LXVIII. THE DIABETOGENIC ACTION OF CRUDE ANTERIOR PITUITARY EXTRACTS

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(*Received 25 January 1938*)

The demonstration by Houssay & Biasotti [1931] that in a number of different species the symptoms of diabetes which normally follow pancreatectomy are greatly reduced by removal of the pituitary gland, has received ample confirmation. Houssay and his colleagues (whose work was summarized by Houssay [1936]) were further able to show that when crude anterior lobe extracts were administered to hypophysectomized-depancreatized animals, the symptoms of diabetes were greatly exacerbated, and that intact animals could be rendered diabetic by the injection of such extracts. This diabetogenic action of anterior lobe extracts in normal animals has also been demonstrated by other investigators [Evans *et al.* 1932; Baumann & Marine, 1932; Evans, 1933; Barnes & Regan, 1933; Nelson & Overholser, 1934], although negative results have been recorded in many instances [Shpiner & Soskin, 1934; Holden, 1934; Hrubetz, 1935; Long, 1936]. Houssay [1936] distinguishes two types of hyperglycaemic action exerted by anterior lobe extracts in intact animals; one, an immediate blood sugar-raising action [Eidelsberg, 1932; Lucke *et al.* 1933; Holden, 1934; Shpiner & Soskin, 1934; Elmer *et al.* 1937] and the other—the true diabetogenic action—exhibited only after repeated daily injections. The discovery that the administration of anterior lobe extracts can render normal animals diabetic has led to the suggestion that the pituitary gland elaborates a "diabetogenic hormone", although there is no evidence to show whether this represents one, or more than one substance. Moreover, the use of the term "hormone" is most inapt, since the normal function of the factor concerned cannot be the production of a diabetic condition.

Because of the numerous negative findings, and because, in successful demonstrations, the true diabetogenic action of anterior lobe preparations is exhibited only after several daily administrations of large amounts of extract, the physiological significance of the diabetogenic action of these extracts in normal animals has recently been questioned [Chaikoff, 1936]. The investigations recorded in the present paper were undertaken in order to determine the conditions necessary for a consistent demonstration of the true diabetogenic action of pituitary extracts. It was hoped thus to throw light on the reason for the negative results recorded in the literature, and to provide a consistent physiological test which might be used in the concentration of the diabetogenic factor. During the course of these investigations it was found that in the dog the daily injection of a crude anterior lobe extract induced, in some cases, a diabetic condition that was apparently permanent. This observation was briefly reported in a preliminary communication [Young, 1937, 2].

The influence of anterior lobe extracts on the glycaemic response to injected insulin [Young, 1936; 1937, 1] is not dealt with in the present communication but is fully discussed in another paper. The present investigation is concerned

[1] Work begun during the tenure of a Beit Memorial Fellowship.

only with the diabetogenic action of anterior pituitary extracts. The criterion of a diabetogenic action adopted has been the induction of hyperglycaemia, glycosuria, ketonuria and polyuria after 3–4 days' daily injection of the extract.

*Methods*

*Estimations.* Urine was collected under toluene from metabolism cages. Urinary sugar was determined by Benedict's method, the reagent being standardized against pure glucose. Urinary ketone bodies were assessed semi-quantitatively on the basis of the Rothera and Gerhardt tests, and the results expressed in units on an arbitrary scale. As shown by estimation of ketone bodies in urine by the Van Slyke-Denigès method, 5 units on the scale are approximately equal to 1 g. of "total acetone". Blood sugar was estimated by a slight modification of the Hagedorn and Jensen method.

*Administration of extracts.* Both the intraperitoneal and subcutaneous routes have been used in these experiments, but the former method of administration seems definitely superior to the latter in the case of the dog, so that in most experiments on this animal this route has been used. The skin is prepared for injection by thorough sterilization with soap and water and alcohol, or mercuric chloride, and a sterile needle is used for injection, which is preferably made through the midline. If such simple conditions are observed a long series of intraperitoneal injections of crude extracts may be made into dogs without the development of the slightest symptoms of infection or ill health; indeed, in many cases, the animal appears to thrive on the injections, in spite of the fact that a large amount of crude extract may be administered daily. Occasionally in dogs mild vomiting follows the first few injections, but usually this phase quickly passes off and later injections occasion no untoward symptoms. In a few cases a superficial skin infection developed in injected dogs, but this usually cleared up without treatment. Three cases of peritonitis, following accidental infection, have occurred in a series of over forty injected dogs; these animals were killed immediately infection was diagnosed and the results from these experiments are not included in the data presented. The cat resembles the dog in reacting better to intraperitoneal injection, but with the other species used the subcutaneous route seems preferable.

*Care of animals.* In these experiments the proper care of animals is vitally important. Houssay [1936] has pointed out that fasting causes the diabetic condition engendered by anterior pituitary extracts to disappear. In the present experiments it has become increasingly clear that unless the experimental animal is in good health and has a normal appetite, results are unsatisfactory. As dogs have been used for long periods in many of the experiments, it has been considered advisable to allow the animals adequate exercise outside the metabolism cages with consequent risk of urine loss. Training of the animals has ensured that the losses have been minimal, in some cases negligible, but it is necessary to point out that the figures given for sugar excretion in this paper are minimum values. Young (6–18 months) male dogs have been used whenever possible, but sometimes older animals and bitches have had to be used, although they are generally less satisfactory than young male animals. In all cases adequate time has been allowed for the animals to become accustomed to the diet and surroundings before injections have begun. All animals were provided with food and water *ad lib.* and in many instances the amount of each constituent consumed was determined by weighing. Dogs received a mixture of cooked meat, raw meat, cooked liver and biscuits, and bones, to which were occasionally added cooked fish and milk. Rabbits were given cabbage or carrots with oats

and bran, while cats received milk, fish and cooked meat. The other species used received appropriate mixed diets.

*Preparation of extracts.* Unless otherwise stated all extracts were prepared from ox pituitary glands removed from the carcass at the slaughter-house within an hour of the death of the animal. Immediately after the glands had been removed from the animal they were frozen in solid carbon dioxide and transported to the laboratory in this state. The glands were dissected in a semi-frozen condition, a clean separation being effected between posterior and anterior lobes. For "crude saline extracts" the anterior lobes were minced while still frozen and ground in the cold store with sand and 2 ml. cold saline/g. of fresh tissue. After 1 hr. at 0° the mixture was centrifuged in chilled pots at about 1500 r.p.m. for 5 min., and the thick supernatant fluid poured off and stored at 0° without freezing. The residue was discarded. The extract was always used within 5 days of preparation.

An alkaline extract of fresh gland was prepared by grinding the fresh frozen anterior lobes in the cold store with cold saline at *p*H 8·5 (faint blue tint with thymol blue) in the proportion of 3 ml./g. of fresh tissue, the *p*H being adjusted and maintained by the frequent addition of small amounts of *N*/5 NaOH. The extraction was allowed to proceed overnight in the cold store, and next morning the residue was centrifuged off and discarded. An acid extract was prepared similarly by extraction with cold saline at *p*H 2·5. The *p*H of the extract was adjusted to 8·5 before injection. Alkaline and acid extracts of fresh gland were stored at 0° for not longer than 5 days before injection.

In the preparation of extracts of fresh gland the residue remaining after one extraction with neutral, acid or alkaline saline has always been discarded. If *X* g. of anterior lobe tissue were used, the extract obtained is described as "an extract of *X* g. of fresh gland", abbreviated for convenience to "*X* g. equivalent of extract". It is clear that such an amount of extract does not contain all the extractable material in *X* g. of gland, owing to the undetermined loss in the discarded residue. If one assumes that anterior lobe tissue contains 80 % water, then the loss of extractable material by the above method of extraction is about 30 % of the total.

Acetone-dried gland was prepared by mincing the dissected gland into 20 vol. of ice-cold dry acetone, and filtering and drying the dehydrated tissue after 24 hr. extraction. The desiccated gland was stored at 0° *in vacuo*; in this condition the diabetogenic activity was retained for at least 1 week. This material was extracted by stirring at room temperature with 7·5 ml. of saline/g. at *p*H 8·5 or 2·5 for 1 hr., the *p*H being maintained by the frequent addition of small amounts of *N*/5 NaOH or HCl; the extraction was repeated after centrifuging and the centrifuged extracts combined, that made by acid being adjusted to *p*H 8 before injection. The acid or alkaline extracts of acetone-dried gland were prepared freshly each day immediately before injection.

## *Results*

Although the blood sugar of injected animals has been determined at intervals, more attention has been paid to the daily sugar excretion. The extent of the daily glycosuria is a more satisfactory indication of the defective metabolism of sugar over 24 hr. than are isolated blood sugar estimations. Determination of the blood sugar has always shown glycosuria to be accompanied by marked hyperglycaemia.

*Experiments with mice, rats and guinea-pigs.* Numerous experiments on the injection of a crude saline extract into mice, rats and guinea-pigs have yielded

unsatisfactory results. Rats received 1 g. equivalent of crude saline extract subcutaneously each day. About 10% of these exhibited slight glycosuria (0·5% of sugar), but comparable results were obtained with control animals receiving a similar extract of fresh rabbit liver. Groups of 6 rats injected daily for periods up to 3 weeks showed no significant rise in mean blood sugar level [cf. Richardson & Young, 1937]. Similar results were obtained with mice. Guinea-pigs exhibited ketonuria when a crude extract was administered subcutaneously (2 g. equivalent/day of crude extract), but glycosuria did not appear. In all three species the urine excretion and food intake of the injected animals were usually subnormal. It was clear that animals of these species would not serve satisfactorily as test animals for the consistent demonstration of diabetogenic activity.

*Experiments with rabbits.* In view of the striking results of Baumann & Marine [1932], who were able to induce a glycosuria of as much as 35 g./day in a rabbit by the daily administration of a small amount of crude gland extract, the results recorded here are very disappointing. In a series of experiments in which crude saline extract (1–3 g. equivalent each day) was administered subcutaneously or intraperitoneally for long periods to nearly 100 rabbits, the maximum sugar excretion encountered was 13·5 g./day, while about 25% of the animals did not excrete sugar at all. No difference in reaction was observed between young animals and adults, or between does and bucks; nor was any difference found between the following strains: Dutch, Himalayan, Belgian hares and Sandy lop-eared. In contrast to the results of Baumann & Marine [1932] polyuria did not result from the administration of crude extracts to rabbits; in most instances a diminution of urine volume was observed, and even in those animals in which definite glycosuria occurred no marked polyuria was exhibited. Ketonuria frequently occurred, but was never very striking.

Baumann & Marine do not state by what route their extract was administered to rabbits. In our experiments both the subcutaneous and intraperitoneal methods of injection were used, but in both instances the injected animals appeared to suffer from some degree of shock with consequent diminution of food intake. In control experiments in which a saline extract of fresh tissue other than the pituitary gland was injected into rabbits glycosuria (max. 1·8 g./day) occurred in 2 of 8 injected animals.

Our results with rabbits are the more surprising in that these animals exhibit marked resistance to the hypoglycaemic action of insulin after administration of anterior pituitary extracts [Cope & Marks, 1934; Young, 1936].

It is clear from the foregoing results that the rabbit, although more satisfactory than the mouse, rat or guinea-pig, is not entirely suitable as a test animal for the exhibition of diabetogenic activity.

*Experiments with cats.* In a series of 8 experiments in which 2–6 g. equivalent of crude saline extract were injected intraperitoneally or subcutaneously into cats each day, four instances of glycosuria were observed, the maximum sugar excretion being 6·4 g./day. The urine volume was usually slightly increased though marked polyuria was absent. Ketonuria was slight. Cats did not take kindly to the experimental conditions or to the administration of extract, and were not considered suitable as test animals.

*Experiments with dogs.* The results of experiments with dogs are in welcome contrast with those of other species. Of 25 animals injected with suitable extracts, only one failed to exhibit glycosuria, ketonuria and polyuria. In most instances the diabetic symptoms were of remarkable intensity, a glycosuria of 10 g./kg. body weight/day frequently being recorded.

(*a*) *Injection of crude saline extracts of fresh gland.* In this group the only negative result in 12 experiments was that with a pregnant bitch, which failed to respond to a preparation of extract which exhibited striking diabetogenic activity on injection into a normal male animal. The results of a typical experiment are given in Fig. 1. The figures on the arrows give the weight of fresh

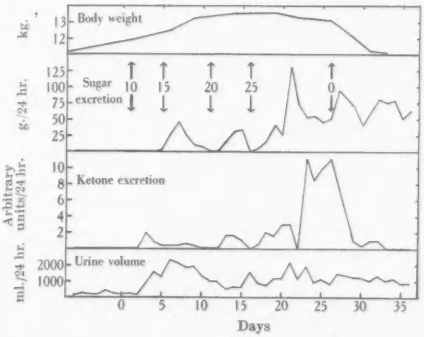


Fig. 1.

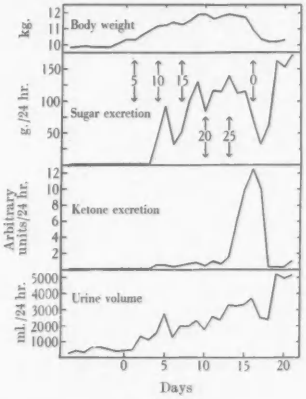


Fig. 2.

Fig. 1. Dog. The figures on the arrows give the weight, in g., of fresh ox anterior lobe used to prepare the amount of extract injected daily, from the day indicated by the arrow, onwards. Daily injections ceased on the day indicated by the arrow carrying 0.

Fig. 2. Dog. The figures on the arrows give the weight, in g., of fresh ox anterior lobe used to prepare the amount of extract injected daily, from the day indicated by the arrow, onwards. Daily injections ceased on the day indicated by the arrow carrying 0.

anterior lobe in g. used to prepare the amount of extract injected daily, from the day indicated by the arrow, onwards. The daily intraperitoneal administration of 10 g. equivalent of saline extract was begun on the first day of experiment, after an initial control period. Polyuria and ketonuria appeared on the 3rd day but the excretion of sugar did not begin until the 5th day of injection when the daily pituitary injection was increased to 15 g. equivalent of extract. During the daily injection of 15 g. equivalent of extract the glycosuria (Fig. 1) increased until the 7th day of injection after which it began to decrease, finally disappearing on the 11th day, at which time the urine had become free from ketone bodies. It is interesting to note that at this time the daily urine volume was still much above the pre-injection level. The phenomenon of the disappearance of the symptoms of diabetes despite the continued daily administration of that dose of extract which induced the appearance of diabetes, has occurred in all animals showing a diabetic response, including species other than the dog. If the daily administration of 15 g. equivalent of extract (Fig. 1) had continued from the 11th day onwards it seems most improbable that glycosuria would have reappeared. Nevertheless when the daily dose was increased to 20 g. equivalent, on the 11th day glycosuria and ketonuria reappeared, to fall again to zero on the 16th day of injection.

It was clearly of interest to determine if a daily level of injection could be reached which was of sufficient magnitude to prevent the animal becoming resistant to the diabetogenic action of the extract. With this object the daily dose was increased to 25 g./day on the 16th day of injection. This resulted in

the reappearance of glycosuria which rose to 130 g./day on the 21st day, thereafter declining to about 50 g./day without showing any tendency to diminish further. On the 24th day the ketonuria reached a high level, and on the 26th day the animal passed into a semi-comatose condition. At this time the $CO_2$-combining power of the blood was 29 vol. % compared with the normal value for the dog of 40 vol. %. Injections were stopped on this day and the ketonuria subsequently rapidly decreased, but the glycosuria persisted, and, 4 weeks after injections had ceased, reached 264 g./day. The subsequent history of this animal has been described elsewhere [Young, 1937, 2].

For the purpose of detection and possible assay of the diabetogenic activity of the extract it was obviously of advantage to determine conditions in which the symptoms of diabetes were present continuously. With this object, a dog (Fig. 2) received 3 daily injections of 5 g. equivalent followed by 3 daily injections of 10 g. The daily injection was increased to 15 g. on the 7th day, to 20 g. on the 10th day and to 25 g. on the 13th day. Injections ceased on the 16th day. By thus rapidly increasing the dose the animal was induced to remain continuously diabetic. This animal remained diabetic after injections were stopped and his subsequent history will be described elsewhere.

(*b*) *Injection of alkaline or acid extracts of fresh gland.* Experiments with acid or alkaline extracts of fresh gland gave results similar to those for saline extracts, and need not be described here. Extracts prepared in this way appeared to be no more potent than those obtained by neutral saline extraction.

(*c*) *Injection of alkaline or acid extracts of acetone-desiccated gland.* Acid or alkaline extracts prepared from dried gland appear to be somewhat less potent than those obtained from fresh gland, but nevertheless were able to induce the appearance of the symptoms of diabetes. In Fig. 3 are shown the results of a

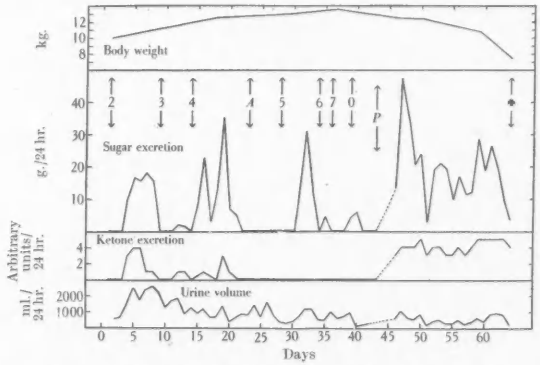


Fig. 3. Dog. The figures on the arrows give the weight, in g., of acetone-desiccated ox anterior lobe used to prepare the amount of extract injected daily, from the day indicated by the arrow, onwards. The arrow carrying *A* indicates the substitution, for a short period, of an acid extract for an alkaline one. The arrow carrying *P* indicates the day on which the dog was depancreatized. The dog died on the 64th day of the experiment.

typical experiment with an alkaline extract of acetone-desiccated gland. The figures on the arrows indicate the weight of desiccated gland (approximately 20 % of the weight of the fresh gland) in g. used to prepare the amount of extract injected daily. From the 24th to the 28th days inclusive an acid extract (of 4 g. of desiccated gland) was injected daily instead of an alkaline one; the lack of

response to this substitution, together with the reappearance of glycosuria when an alkaline extract of 5 g. of desiccated gland was administered, indicates that the acid extract was probably no more potent than the alkaline preparation. On the 45th day after pituitary injections were begun, this dog was anaesthetized and the pancreas was removed aseptically. He made a rapid recovery from the operation, but began to lose weight rapidly and died 21 days after pancreatectomy. This rapid loss of body weight is surprising inasmuch as the glycosuria and ketonuria were relatively small, and the animal's appetite good. The completeness of the pancreatectomy was verified at autopsy when the liver fat content was found to be 31 %.

When the daily dose of an alkaline extract of desiccated gland was rapidly increased (Fig. 4) the diabetic condition disappeared but subsequently returned.

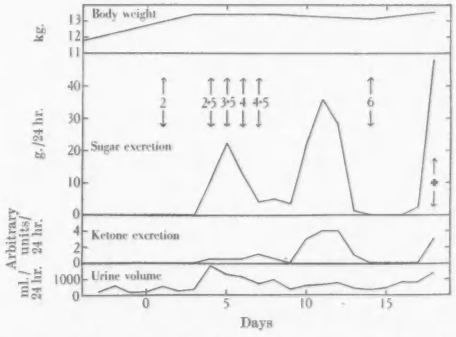


Fig. 4. Dog. The figures on the arrows give the weight, in g., of acetone-desiccated ox anterior lobe used to prepare the amount of extract injected daily, from the day indicated by the arrow, onwards. The dog was killed on the 18th day of the experiment.

This animal was killed on the 18th day of injection at which time the sugar excretion was 48 g./day. The liver glycogen content was found to be 1·4 % and the liver fat 7·8 %.

(*d*) *Control experiments with dogs.* As the diabetogenic action of anterior pituitary preparations is shown in normal animals only after the injection of a large amount of crude extract, often into the abdomen, it was desirable to carry out rigid control experiments. Those performed fall into two classes: (1) those with non-diabetogenic anterior pituitary preparations; (2) those with a crude extract of tissues other than the anterior pituitary gland.

Experiments in the first class were made with extracts of commercial dried anterior lobe or with extracts of fresh gland that had been kept at room temperature for 12 hr. or more. The methods of extraction used for these control experiments were similar to those used for the preparation of active extracts, and the volume of extract injected was similar to that used in previous experiments. In no case was glycosuria or ketonuria induced, the only observed effect of the injections being a striking rise in daily urine volume, in some cases to 2–3 l. per day. This diuretic action of anterior pituitary extracts has been previously observed by other workers [Teel, 1929; Barries *et al.* 1933]. In the present investigations polyuria without glycosuria was induced by anterior pituitary injections only in cats and dogs. In the other species investigated the urine excretion showed a tendency to fall rather than to rise.

The intraperitoneal injection of crude extracts of rabbit muscle or liver apparently had no effect whatever on our dogs. In Table I is shown the result of an experiment in which an extract of rabbit muscle was injected intraperitoneally into a dog. It will be seen that such an extract has no diuretic action, and had no visible effect on the animal whatever.

Table I. *Daily intraperitoneal injection of a crude extract of rabbit muscle into a dog*

| Day | Daily urine vol. ml. | Urinary sugar | Urinary ketone | Amount of extract injected daily (g. of fresh tissue) | Wt. kg. |
|---|---|---|---|---|---|
| Control period | Av. = 200 | 0 | 0 | 0 | 9·40 |
| 1 | 270 | 0 | 0 | 10 | — |
| 2 | 365 | 0 | 0 | 10 | — |
| 3 | 135 | 0 | 0 | 10 | — |
| 4 | 175 | 0 | 0 | 15 | — |
| 5 | 275 | 0 | 0 | 15 | — |
| 6 | 200 | 0 | 0 | 15 | — |
| 7 | 280 | 0 | 0 | 20 | — |
| 8 | 190 | 0 | 0 | 20 | — |
| 9 | 310 | 0 | 0 | 20 | 10·20 |

Such control experiments suggest that the diabetogenic action of a crude anterior lobe extract cannot be ascribed to a non-specific action of a voluminous intraperitoneal injection of crude protein solution.

## Discussion

If the criteria of the existence of a diabetic condition are considered to be the exhibition of hyperglycaemia, glycosuria, ketonuria, polyuria and polydipsia, then of the species examined in the present investigation only the dog (and possibly the cat) developed diabetes in response to the anterior pituitary injections. In the other species, polyuria, together with polydipsia and polyphagia were usually absent. It is remarkable that dogs exhibiting a profound glycosuria have lost little or no weight in the present experiments, differing in this respect from depancreatized animals and from many clinical cases of diabetes mellitus.

The relative insensitivity of species other than the dog to the diabetogenic action of crude anterior lobe extracts may account for many failures to confirm Houssay's original finding. The present investigation certainly provides strong support for Houssay's contention that the anterior pituitary lobe contains a diabetogenic substance effective in intact animals. The reason for the marked species difference is not clear. It is interesting that the two species found to give the best results (cat and dog) are both carnivorous, although no great significance can be attached to this observation at present. It may be emphasized here that the dogs used in this investigation have received a normal, high protein diet, and it has proved unnecessary to provide a high proportion of carbohydrate food in order to make the effects of the pituitary extracts manifest. Our failure to confirm the results of Baumann & Marine with rabbits is puzzling, and there appears to be no simple explanation at present. It seems improbable that a difference in the type of rabbit used can account for such a marked difference in results. Furthermore, a number of different strains of rabbit have been found, in the present investigations, to give similar results when injected with extract.

Houssay has repeatedly stressed the importance of using fresh glands for the preparation of diabetogenic extracts, and of preparing and storing the extract at

low temperature. In the present investigation these points have been found to be of paramount importance, and it seems that unless there is available a supply of fresh glands frozen immediately after removal from the animal, satisfactory results cannot be expected. It is clear, however, from the present investigation, that the diabetogenic activity of fresh gland is not lost when the tissue is desiccated in cold acetone and stored for 1 week *in vacuo* at 0°.

The disappearance of the symptoms of diabetes in dogs and other animals despite the continued daily administration of a fixed amount of extract, together with their reappearance when the daily dose is increased, is of great interest. The production of an "antihormone" of the type postulated by Collip [1934] seems improbable because of the rapidity of the reaction. Pancreatic tissue removed from a dog which had become resistant to the diabetogenic action of the extract after displaying an initial response was examined histologically by Dr K. C. Richardson, who reported unusual mitotic activity in the islet cells. It therefore seems possible that the animals are able to develop a resistance to the diabetogenic action of the extract because the pancreatic islet tissue becomes hypertrophic, with consequently enhanced insulin secretion. The question of the action of anterior pituitary extracts on pancreatic islets has been further investigated and is the subject of another communication [Richardson & Young, 1937]. If the diabetogenic factor in anterior pituitary extracts is accompanied by a more slowly acting "pancreotropic substance" acting antagonistically via the pancreas, then the results with dogs might receive a rational explanation. Moreover, the relative ineffectiveness of diabetogenic extracts in other species might result from their rapid and effective response to the pancreotropic substance. Such a suggested explanation does not require the assumption that the diabetogenic factor or the pancreotropic substance are separate entities, nor does it entail that the diabetogenic factor should act only by an extra-pancreatic mechanism.

Collip [1935] has described an experiment in which a depancreatized dog, maintained on insulin, was treated with an anterior pituitary extract rich in both growth and ketogenic principles, for a period of 4 months, after which insulin therapy was stopped. For a period of 1 month thereafter the animal showed no increase in acetonuria, although marked hyperglycaemia and glycosuria were present. In an attempt to obtain similar results in the present investigation the animal, whose curves are given in Fig. 3, was depancreatized 42 days after daily injections of crude anterior pituitary extract had begun. Although ketonuria had been absent for 21 days before pancreatectomy, i.e. the animal had become resistant to the ketogenic action of the anterior pituitary extract, a substantial ketonuria appeared after removal of the pancreas. The animal rapidly lost weight and died 21 days after pancreatectomy, giving no evidence of the presence in its blood of an antihormone to the diabetogenic or ketogenic substances of the anterior pituitary gland.

Houssay [1936] distinguishes two types of hyperglycaemic action of anterior lobe extracts, one, an immediate blood sugar-raising action and the other—the true diabetogenic action—exhibited only after repeated daily injections. As Serio [1936] and Houssay [1937] have shown, the daily administration of adrenaline, which has a marked and immediate hyperglycaemic effect, does not produce a diabetic condition in dogs. The results of the present investigations are in entire agreement with Houssay's views. The administration of the diabetogenic extracts (1 g. equivalent/kg. body wt.) used in the present experiments has no marked immediate effect on the blood sugar level of the injected animal, the general tendency being a slight fall of about 10 mg./100 ml. during 2–3 hr.

following injection. Nevertheless, if such a fresh pituitary extract is allowed to remain at room temperature for several hours, it loses its true diabetogenic activity and may simultaneously acquire the ability to cause hyperglycaemia immediately after injection. One is perhaps not justified in saying on this basis alone that the immediate blood sugar-raising action of pituitary extracts is of no physiological significance, although such may well be the case. The injection of a fresh, crude extract of posterior pituitary lobe can cause an immediate rise of blood sugar level, and contamination of anterior lobe extract with posterior lobe principles may account for some of the published results in which anterior lobe extracts were found to exert a rapid hyperglycaemic action.

It seems necessary again to emphasize the need for greater precision in the use of the term "diabetogenic". This description should be applied only to those extracts which possess what Houssay describes as a "true diabetogenic activity", i.e. their injection has little or no immediate effect on the blood sugar level, although continued daily administration induces, after a latent period of some days, a state of hyperglycaemia, glycosuria, ketonuria, polyuria and polydipsia. The active principle or principles concerned should be described as the "diabetogenic factor" and not as the "diabetogenic hormone". Investigations in which the injection of an anterior pituitary extract is shown to induce nothing more than a rise of blood sugar level within a few hours of injection, should not be described as demonstrating a "diabetogenic action" of the pituitary preparation. Extracts having nothing more than this type of effect on the blood sugar level should be described as "hyperglycaemic" and not "diabetogenic". This discussion is relevant to a recent paper of Elmer *et al.* [1937]. These authors injected *intravenously* into rabbits an acid extract of 1 g./kg. body wt. of dried anterior lobe (i.e. weight for weight 5 times the initial dose used in the present investigation), finding a rise of blood sugar varying between 250 and 30 mg./100 ml. during the following 3 hr. No control experiments using tissue extracts other than anterior pituitary preparations are described. Despite the fact that the recognized symptoms of diabetes mellitus include, beside hyperglycaemia, glycosuria, ketonuria, polyuria and polydipsia, and despite Houssay's views, quoted above, these results are described as a demonstration of the diabetogenic action of anterior lobe extract, in agreement with Houssay's findings. On this basis, extracts of the adrenal medulla and of the posterior pituitary lobe contain far more diabetogenic factor than those of the anterior pituitary gland, while a partially autolysed non-specific tissue extract containing peptone might be judged as an especially rich source.

One may conclude, however, from the results of the present investigation that the normal male dog is an excellent test animal for the examination of the true diabetogenic action, in intact animals, of anterior pituitary extracts. The application of this finding to preliminary attempts to fractionate diabetogenic extracts is described in the following paper.

## Summary

1. The diabetogenic action of crude ox anterior lobe extracts has been investigated in the following species: mouse, rat, guinea-pig, rabbit, cat and dog.

2. The mouse, rat and guinea-pig appear to be almost completely insensitive to the diabetogenic action of an extract which is effective in other species. With rabbits and cats, glycosuria and ketonuria followed the daily injection of a crude extract in 25–50 % of the cases investigated, but with dogs, only one failure (in a pregnant bitch) was recorded in 25 experiments.

3. In the dog, glycosuria, hyperglycaemia and ketonuria appear only after 3 or 4 daily injections of extract, and may then disappear within 4 or 5 days in spite of the continued daily injection of the same dose of extract. The diabetic symptoms reappear, however, if the daily dose is suitably increased, and may again subside, only to be revived by a further increase in the amount of extract injected daily. If a sufficiently large daily injection be given the diabetic condition may continue indefinitely after cessation of injections [Young, 1937, 2].

4. Preparation and conservation of the extract at low temperature are essential for the demonstration of diabetogenic activity. Acetone-desiccation of the fresh gland appears to diminish, without entirely destroying, its diabetogenic activity.

5. With dogs, a non-glycosuric polyuria (up to 300 ml./kg./day) may be induced by the daily injection of anterior lobe extracts of which the diabetogenic factor has been inactivated. Such a polyuric action of crude extracts was not observed in mice, rats, guinea-pigs or rabbits.

6. The diabetogenic extracts used in the present investigation do not cause any marked rise of blood sugar immediately after injection. The importance is stressed of differentiating clearly between a true diabetogenic action exhibited only after 3 or 4 daily injections of extract, and the action of other types of extract in causing a rise of blood sugar level within a few hours of administration. The name "diabetogenic factor" (not hormone) should be reserved for the substance (or substances) present in anterior pituitary extracts, which possesses true diabetogenic activity and has little or no action on the blood sugar level within a few hours of injection.

At University College, during the early part of this investigation, I was in receipt of a grant from the Medical Research Council, which partly defrayed expenses; at this time a grant from the Diabetic Association provided for technical assistance. I wish to express my sincere thanks for these grants, and also for the valuable technical assistance of E. A. Woollett.

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# LXIX. STUDIES ON THE FRACTIONATION OF DIABETOGENIC EXTRACTS OF THE ANTERIOR PITUITARY GLAND

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In a series of short communications the author has presented evidence to show that a factor in anterior pituitary extracts, often associated with prolactin, can abolish the hypoglycaemic action of insulin in intact rabbits [Young, 1936, 1, 2; 1937, 1, 2]. The name "glycotropic factor" [Young, 1936, 2] was suggested as a convenient provisional designation for this substance, not because it is believed that this factor is different from all the known pituitary hormones, but because, until the question of whether or not this substance antagonizes all the known actions of insulin is settled, the description "anti-insulin" or "contra-insulin" substance is unsatisfactory. Evidence has been brought forward to show that the glycotropic factor is not identical with the gonadotropic or thyrotropic hormones or with prolactin [Young, 1937, 1], although closely associated with preparations of the latter, but the question of whether or not it is identical with any of the other hormones of the anterior pituitary gland must at present remain open. More recently other investigators have found that the injection of preparations of prolactin may influence the blood sugar level of injected animals [Long, 1937, 2; Riddle, *et al.* 1937]. Indeed the latter authors suggest that prolactin has a diabetogenic action. On the other hand, Nelson *et al.* [1935] found that injections of prolactin did not induce the appearance of diabetic symptoms in monkeys or rabbits.

The author has stressed the fact that although two injections of prolactin can, under suitable circumstances, completely abolish any hypoglycaemic response to the subsequent intravenous administration of 2 units of insulin in the intact rabbit, the blood sugar level of the injected animal is not materially affected thereby, marked hyperglycaemia being absent [Young, 1936, 1, 2; 1937, 2]. The present investigation is concerned only with the diabetogenic action of pituitary extracts, and the question of the non-hyperglycaemic insulin resistance in prolactin-injected rabbits will be discussed in a subsequent communication. The fact that prolactin administration can induce insulin resistance in rabbits, together with the observation that prolactin injections maintain a high glycogen level in fasting rabbits [Young, 1937, 1] suggested the necessity of investigating thoroughly the possible diabetogenic action, in this species, of prolonged daily prolactin injections. However, in the preceding paper it has been shown that the rabbit is not a satisfactory animal for the demonstration of diabetogenic activity, although the dog is suitable in this respect. It was therefore of importance to determine whether or not prolactin is diabetogenic in the dog. The investigations described in the preceding paper showed that preparation

[1] Work begun during the tenure of a Beit Memorial Fellowship.

and conservation of a crude extract at 0° are essential for the demonstration of diabetogenic activity, so that it was necessary to investigate the possible diabetogenic properties of prolactin prepared and stored at 0°. The corresponding "non-prolactin" fractions were also tested.

In the preceding paper experiments were described in which large doses of a crude extract of commercial dried gland failed to induce diabetes on administration to dogs. As these crude extracts were rich in thyrotropic activity it seemed improbable that the thyrotropic hormone *per se* could be diabetogenic. Nevertheless, it seemed advisable to exclude the thyrotropic hormone completely by testing a partially purified preparation of thyrotropic hormone, free from prolactin.

In connexion with another investigation, young rhesus monkeys were given daily injections of ordinary prolactin for long periods. Opportunity was taken to determine any effect of these injections on the blood sugar level.

## *Methods*

The general methods are those of the preceding paper.

*Preparation of extracts.* The preparations of prolactin used in the present investigation all possessed glycotropic activity, and the term "prolactin" is used to designate a substance having the ability to cause growth of the pigeon crop-gland [Riddle & Braucher, 1931] and the power to abolish, in suitable conditions in the rabbit, the hypoglycaemic action of injected insulin [Young, 1936, 1, 2]. Although prolactin free from glycotropic activity was not used in the present investigation, an extract containing glycotropic activity but no demonstrable prolactin was tested.

(1) "*Ordinary prolactin*", *i.e. prolactin extracted from commercial* (*B.D.H. Ltd.*) *dried gland and fractionated at room temperature.* Ordinary prolactin was obtained from acetone-dried ox anterior lobe by a modification of the aqueous alcohol extraction method of Bates & Riddle [1935]. The modification consisted in the substitution of the fractionation with 70% alcohol in the presence of NaCl by a large number of precipitations from water at *p*H 5·5. After 6–12 such reprecipitations the prolactin fraction was found to be free from $Na_2SO_4$, and to contain no detectable gonadotropic or thyrotropic activity as determined by the rabbit ovulation and guinea-pig thyroid hyperplasia tests, respectively. This prolactin fraction will be described as "ordinary prolactin". The fraction soluble at *p*H 3–4 in the presence of $H_2SO_4$ and $Na_2SO_4$, which contained the thyrotropic and gonadotropic hormones, was dialysed (Visking cellophane) against distilled water at 2° until free from $SO_4^{=}$. The precipitate, which formed during dialysis, was spun off, and the soluble material precipitated with alcohol and dried. This fraction constituted "ordinary non-prolactin". A number of preparations of prolactin were obtained from dried gland by the simple *p*H 5·5 precipitation method. The experimental results with material prepared in this manner were similar to those with "ordinary prolactin", although it was not possible to free the prolactin fraction from thyrotropic and gonadotropic activities by this procedure.

(2) "*Prolactin-C*", *i.e. prolactin extracted at 0° from fresh tissue and fractionated at low temperature.* The fresh ox glands were collected and dissected as described in the previous paper. The minced, frozen tissue was ground with sand and 3 ml./g. of cold saline in the cold store; *N*/5 NaOH was stirred in until the *p*H was about 8·5 (faint green with thymol blue) and the mixture left overnight in the cold store. All subsequent procedures were carried out at a temperature as

close as possible to 0°. The next morning the *p*H was again adjusted to 8·5 if necessary and the mixture centrifuged in chilled pots for 10 min. at 3000 r.p.m. The thick extract was decanted and the residue discarded. The *p*H of the extract was adjusted to 5·5 (faint pink with chlorophenol red) with *N*/5 HCl and the mixture again centrifuged. The clear, supernatant fluid was then pipetted off and the precipitate stirred at *p*H 8·5 with a volume of cold saline equal to that originally used for extraction. After 30 min. stirring, the *p*H was adjusted to 5·5 and the mixture again centrifuged. Four precipitations of the material insoluble at *p*H 5·5 (prolactin) were effected in this way. The final prolactin precipitate was stirred with cold saline at *p*H 8·0, the total volume being equal to that of the saline used originally for extraction. This constituted the "prolactin-C" preparation. A clear solution at *p*H 8 was not obtained owing to the presence of much colloidal matter. The clear supernatant liquid, from the first precipitation of the prolactin at *p*H 5·5, was adjusted to a volume equal to the final volume of the prolactin fraction by addition of a part of the supernatant liquid from the second prolactin precipitation, and the *p*H adjusted to 8·0. This constituted the "non-prolactin-C" fraction. Both the prolactin and the non-prolactin fractions were stored at 0° and used within 4 days of preparation.

The amounts of "prolactin-C" and "non-prolactin C" are expressed in terms of the number of g. of fresh gland used to provide the given amount of extract. As with the crude saline extract of fresh gland, an extract of *X* g. of fresh tissue (abbreviated for convenience to *X* g. equivalent of extract) does not contain all the extractable material in *X* g. of gland owing to the undetermined loss in the discarded residues.

For the purposes of rough comparison it may be stated that 1 mg. of ordinary prolactin is equivalent to about 60 mg. of fresh gland.

*Biological tests.* Prolactin was assayed by the pigeon crop-gland method as described by Rowlands [1937]. Thyrotropic hormone was estimated by the method of Rowlands & Parkes [1934]. Gonadotropic activity of ox pituitary extracts was assayed by ability to cause ovulation in the oestrous rabbit. Glycotropic activity was determined on rabbits by a modification of the method previously described [Young, 1936, 1].

## Results

### (I) *General biological assays*

The results of assays for prolactin (Table I), thyrotropic hormone (Table II) and gonadotropic hormone (Table III) on typical preparations used in the present investigation, are given in the following tables.

Table I. *Prolactin assays by the method of Rowlands [1937]*

| Substance | Amount injected daily | Increase in crop-gland per 300 g. pigeon—av. of 10 birds g. |
|---|---|---|
| "Ordinary prolactin" | 1 mg. | 2·7 |
| "Ordinary non-prolactin" | 20 mg. | 0 |
| "Prolactin-C" | 0·033 g. equivalent of fresh tissue | 1·4 |
| "Non-prolactin-C" | 0·50 g. equivalent of fresh tissue | 0 |

Table II. *Thyrotropic hormone assays by the method of Rowlands & Parkes [1934]*

| Substance | Amount injected daily | Increase in thyroid weight—av. of 10 cavies mg. |
|---|---|---|
| "Ordinary prolactin" | 20 mg. | − 3·5 |
| "Ordinary non-prolactin" | 1 mg. | +32·5 |
| "Prolactin-C" | 0·17 g. equivalent of fresh tissue | + 5·0 |
| "Non-prolactin-C" | 0·17 g. equivalent of fresh tissue | +43·0 |

Table III. *Gonadotropic hormone assays*

| Substance | Approximate dose for ovulation in 50% of oestrous rabbits |
|---|---|
| "Ordinary prolactin" | >100 mg. |
| "Ordinary non-prolactin" | 0·6 mg. |
| "Prolactin-C" | 7 g. equivalent |
| "Non-prolactin-C" | 0·17 g. equivalent |

Glycotropic activity was determined as follows. A rabbit was deprived of food at 5 o'clock in the evening and given an injection of the substance under investigation, a similar injection being given at 9.30 a.m. on the following morning. At 2 p.m. 2 units of crystalline insulin were given intravenously and the blood sugar level determined at frequent intervals during the following 4 hr. A typical result is given in Fig. 1. In this instance two injections, each of

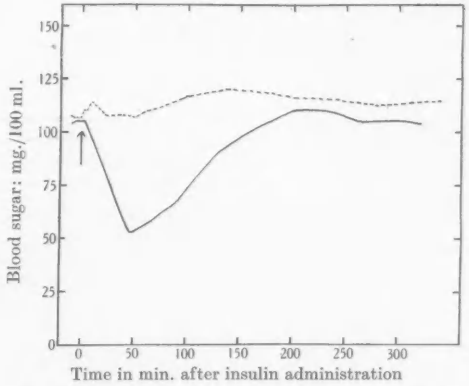


Fig. 1. Effect of two injections of prolactin on the blood sugar response to 2 units of crystalline insulin administered intravenously to the rabbit. Curve (*a*). Response before prolactin injections. Curve (*b*). Response after prolactin injections. The arrow indicates the time of insulin injection. The second prolactin administration had been made 4½ hr. before the insulin injection.

125 mg. of ordinary prolactin, completely abolished the hypoglycaemic effect of 2 units of intravenously administered crystalline insulin, although the initial blood sugar level was not affected. The approximate minimum amounts of different substances required, in similar tests, to abolish completely, or almost completely, the hypoglycaemic action of 2 units of crystalline insulin, are given in Table IV.

Table IV. *Glycotropic factor assays*

| Substance | Minimum dose required for each of two injections to abolish insulin hypoglycaemia in the above test |
|---|---|
| "Ordinary prolactin" | 80–150 mg. |
| "Ordinary non-prolactin"* | 100–180 mg. |
| "Prolactin-C" | About 2 g. equivalent of fresh tissue |
| "Non-prolactin-C"* | About 3 g. equivalent of fresh tissue |

* If the anterior lobe tissue extracted contained any posterior lobe principles, these would be present in this fraction. If present in sufficient amount they would render impossible a test of the glycotropic activity of this fraction by the above method.

The presence of glycotropic activity in the "ordinary prolactin", which contains no detectable gonadotropic and thyrotropic hormones, and in the "non-prolactin fraction" which contains no detectable prolactin, shows that the glycotropic factor cannot be identical with the gonadotropic or thyrotropic substances, or with prolactin.

(II) *The possible diabetogenic action of prolactin in the rabbit*

Rabbits frequently excrete sugar when injected daily for a long period with large doses of ordinary prolactin. Fig. 2 shows the result of a typical experiment

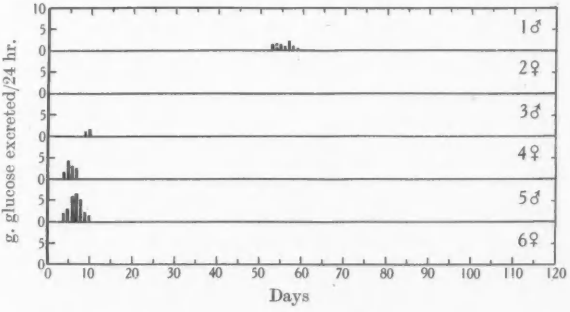


Fig. 2. The daily sugar excretion of rabbits receiving daily injections of 100 mg. of "ordinary prolactin".

in which 6 rabbits (3 ♂ and 3 ♀) were each injected daily with 100 mg. of ordinary prolactin. Although as much as 6·2 g. of sugar were excreted during 24 hr. in one instance, ketonuria and polyuria were absent at all times. Two rabbits did not excrete sugar at any time during an experiment lasting 117 days. Three pregnant rabbits also excreted sugar during the course of similar daily prolactin injections (Fig. 3). It will be seen that there is no obvious relationship between the time of littering and the time of onset of glycosuria. In all these experiments milk appeared in the mammary glands of the adult female rabbits within 1 week of the initiation of daily prolactin administrations.

When "prolactin-C" was administered daily to rabbits for a long time glycosuria occurred once in seven experiments, with a maximum value of 1·9 g./day. This result differs from that for ordinary prolactin, and resembles that for control experiments (preceding paper).

Although the administration of a suitable preparation of prolactin to a rabbit may result in the abolition of the hypoglycaemic action of injected insulin without causing a hyperglycaemia during the course of the experiment, if ordinary prolactin is used in an experiment of this kind, glycosuria may appear

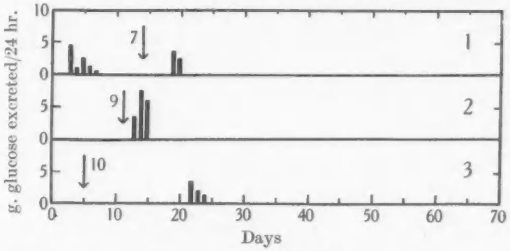


Fig. 3. The daily sugar excretion of pregnant rabbits receiving daily injections of 100 mg. of "ordinary prolactin". The arrows indicate the day of littering, the figures on the arrows giving the number of healthy young in the litters.

2 days or more after the last injection of prolactin. It has never been observed when "prolactin-C" is used. A similar sugar excretion was observed in control experiments in which an extract of rat liver was injected instead of prolactin.

Experiments in which rabbits were given daily injections of "ordinary non-prolactin" and "non-prolactin-C" gave results similar to those for control experiments (preceding paper).

### (III) *Experiments with dogs*

Of four dogs given daily intraperitoneal injections of 400–1000 mg. of ordinary prolactin, none became diabetic. One animal excreted 6·8 g. of sugar on the 4th day of injection, but no other instance of glycosuria was observed in these experiments. Ketonuria and marked polyuria were absent, although a definite increase in urine excretion (to about 800 ml. daily) occurred in some instances. No case of lactation was observed in these male dogs, and in general the injection of large doses of prolactin had little observable effect on them.

Two dogs which received daily intraperitoneal injections of "ordinary non-prolactin", the daily dose rising from 100 to 300 mg., did not exhibit glycosuria at any time, the only observable effect of the injections being a slight ketonuria and polyuria (1000–1400 ml./day). Loss of body weight was not observed. Both animals subsequently became diabetic on receiving daily doses of crude anterior lobe extract containing less than 1/7th of the amount of thyrotropic hormone which had previously been ineffective.

Neither of two dogs injected with "prolactin-C" exhibited glycosuria although the dose injected daily was increased to the equivalent of 25 g. of fresh gland. Fig. 4 illustrates one of these experiments. The figures on the arrows indicate the weight of fresh gland extracted to provide the daily dose of "prolactin-C". At the arrow marked *X* the daily injection of "prolactin-C" equivalent to 25 g. of fresh gland was supplemented by the addition of "non-prolactin-C", also equivalent to 25 g. of fresh gland. Glycosuria appeared on the 4th day of injection of this mixture and rose to 65·4 g./day on the 7th day. A substantial ketonuria was also exhibited. The glycosuria and ketonuria disappeared on the 9th day of injection. This experiment suggested that "non-prolactin-C"

alone might be effective, and this was found to be so. Fig. 5 illustrates one of the two similar experiments carried out in this connexion. In this instance the dog used was a litter-mate of that providing the results for Fig. 3. The effective dose of "non-prolactin-C" is greater than might be expected from the amount of fresh gland used in its preparation, but this is probably due to the unavoidable

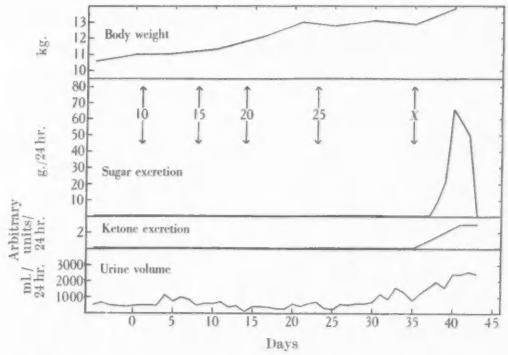


Fig. 4. Bitch receiving daily injections of "prolactin-C". The figures on the arrows give the weight, in g., of fresh ox anterior lobe used to prepare the amount of extract injected daily, from the day indicated by the arrow, onwards. On the day indicated by the arrow carrying *X* the daily injection of "prolactin-C" equivalent to 25 g. of fresh gland was supplemented by the addition of "non-prolactin-C", also equivalent to 25 g. of fresh gland. The animal used in this experiment was the litter-mate of that used in the experiment illustrated in Fig. 5.

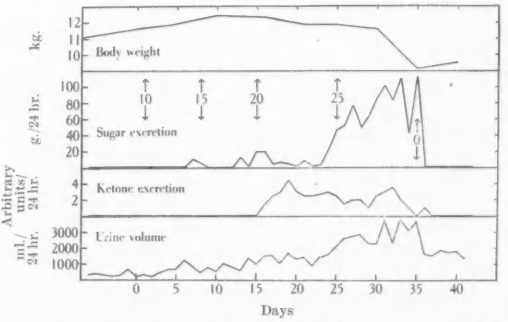


Fig. 5. Bitch receiving daily injections of "non-prolactin-C". The figures on the arrows give the weight, in g., of fresh ox anterior lobe used to prepare the amount of extract injected daily, from the day indicated by the arrow, onwards. Injections ceased on the day indicated by the arrow carrying 0. The animal used in this experiment was the litter-mate of that used in the experiment illustrated in Fig. 4.

losses which occur in the preparation of this fraction. The last injection of "non-prolactin-C" (Fig. 5) was given on the 35th day of the experiment, at which time the sugar excretion was 113 g./day. The urine became sugar-free on the following day and ketonuria disappeared 2 days later.

### (IV) *Experiments with monkeys*

Four young female monkeys were injected daily with 60–200 mg. of ordinary prolactin, for periods ranging up to 9 months. No significant variation was found in blood sugar determinations made at weekly intervals, and at no time were any symptoms of diabetes mellitus observed in these injected animals.

## Discussion

The experiments with dogs indicate that prolactin *per se* is not diabetogenic in the intact animal, and further that diabetogenic preparations may be obtained which contain no detectable prolactin, as assayed by the pigeon crop-gland method. This is perhaps surprising in view of the marked glycotropic activity of the prolactin preparations used. Moreover, prolactin, prepared at low temperature and under conditions which permit the preparation of potent diabetogenic fractions, failed to produce a diabetic condition when given in large doses to dogs.

With rabbits, prolonged administration of prolactin induced, in many instances, a delayed glycosuria, ordinary prolactin being more effective than prolactin-C in this respect. This glycosuria was not accompanied by ketonuria or polyuria, and a condition of diabetes could not be accurately diagnosed. The rabbit apparently differs from the dog in being more prone to exhibit a transient glycosuria in response to injections of prolactin, a difference similar to that found with non-specific, control injections (preceding paper). In a glycotropic test (see above) the blood sugar level of a rabbit receiving injections of prolactin is not markedly affected, although the hypoglycaemic response to injected insulin may be completely abolished. Under the conditions of this test the liver glycogen content of the experimental rabbits is much greater than that of control animals [Young, 1937, 2], and the abolition of the hypoglycaemic action of insulin is probably associated with a rapid and effective response of the hepatic glycogenolytic mechanism to a suitable stimulus. It is found that rabbits which have become insulin-resistant following administration of glycotropic factor respond to a very slight emotional stimulus by a substantial rise of blood sugar. It is therefore not surprising that instances of hyperglycaemia and glycosuria are observed in rabbits after a number of daily injections of prolactin, although it must be emphasized that, in the absence of ketonuria, a diabetogenic action of prolactin is not thus demonstrated. The need for greater precision in the use of the word "diabetogenic" has already been stressed in the preceding paper. What was there said of crude pituitary extracts applies equally to partially purified preparations, viz. that the use of the description "diabetogenic" should be confined to extracts the daily injection of which induces, after a latent period of some days, a state of hyperglycaemia, glycosuria, ketonuria, polyuria and polydipsia. The fact that daily prolactin injections induce, in some rabbits, nothing more than a transitory hyperglycaemia and glycosuria is not, *per se*, evidence of a diabetogenic action of the substance. The negative results with dogs receiving prolactin injections, and the positive finding with prolactin-free preparations, show that prolactin is not the diabetogenic substance of the anterior lobe.

Riddle *et al.* [1937] have recently found that, in pigeons, daily injections of prolactin for 1–8 days increase the blood sugar level by 7–33 % and conclude "thus in pigeons the diabetogenic response is found to be a response to prolactin". Again it must be emphasized that a slightly raised blood sugar level

does not constitute a diabetic condition, and that in prolactin-injected animals a slight emotional stimulus, such as that associated with the taking of blood samples, is very prone to provoke hyperglycaemia.

As prolactin possessing marked glycotropic activity is not truly diabetogenic in the normal rabbit or in the normal dog, it is clear that the diabetogenic factor and the glycotropic substance are not identical. Nevertheless, all the diabetogenic preparations used in the present investigation possessed marked glycotropic activity, and it seems probable that the diabetogenic factor comprehends the glycotropic substance together with another factor or factors.

The foregoing discussion applies only to intact animals. Recently, Long [1937, 1, 2] has found that the sugar excretion, but not the ketonuria, of a hypophysectomized-depancreatized cat is increased by the administration of prolactin-adrenotropic preparations of the pituitary gland. This author [1937, 1] has made the tentative suggestion that the glycosuric substance of the anterior pituitary gland is either prolactin or the adrenotropic hormone. Long [1937, 2] suggests that the diabetogenic factor may be composed of two constituents: one, which is heat-stable, being able alone to precipitate glycosuria but not ketonuria in the hypophysectomized-depancreatized animal, while the presence of the other, which is heat-labile, as well as that of the first, is necessary to produce glycosuria and ketonuria in the intact animal. The relevant results of the present investigation are in entire agreement with Long's view. It seems probable that Long's heat-stable factor which is unable to induce glycosuria and ketonuria in normal animals, is our glycotropic factor. If this is so, then Long's suggestion may be extended to include the supposition that the heat-stable constituent of the diabetogenic complex is not prolactin but a factor which can produce a condition of insulin resistance in the intact animal without any marked effect on the blood sugar level (glycotropic factor).

In the experiments with "ordinary non-prolactin" the amount of thyrotropically active substance injected daily substantially exceeded that given in experiments with "non-prolactin-C". As "ordinary non-prolactin" lacks diabetogenic activity, the observed diabetogenic activity of the "non-prolactin-C" fraction could not have been due merely to its thyrotropic activity.[1] It seems improbable that a combination of the glycotropic factor and the thyrotropic hormone alone is diabetogenic, as this combination was present in the "ordinary non-prolactin" fraction as well as in the extract of commercial, acetone-dried gland, which, in the preceding paper, was found to be ineffective. These considerations do not rule out the possibility that the thyrotropic hormone is an essential constituent of the diabetogenic complex [cf. Barnes & Regan, 1933], but do suggest that the presence of at least one other substance is necessary for the exhibition of diabetogenic activity. The possibility that another factor is the ketogenic substance of the anterior lobe [Burn & Ling, 1930] is an attractive one, and is at present under investigation.

## Summary

1. The daily injection of an alkaline extract of fresh ox anterior pituitary gland, prepared at low temperature, from which the prolactin has been removed, induces the symptoms of diabetes in dogs.

[1] The thyrotropic activity considered here is that which induces hyperplasia of the thyroid gland [cf. Rowlands & Parkes, 1934]. The assumption is usually made that the factor inducing this response is identical with that causing increased secretion of the thyroid gland, but the possibility that more than one factor is concerned cannot be ruled out.

2. The daily injection of large doses of prolactin does not produce diabetes in dogs, even when the prolactin is prepared from fresh glands at a low temperature, under conditions which permit the preparation of active diabetogenic preparations.

3. The conclusion is drawn that neither prolactin nor the glycotropic ("anti-insulin") factor closely associated with, but distinct from it, is diabetogenic in intact dogs.

4. The administration of prolactin to rabbits or monkeys does not induce a diabetic condition, although rabbits may exhibit a transitory glycosuria. This transitory glycosuria cannot correctly be described as a diabetic condition since it is not accompanied by ketonuria or polyuria.

5. The daily administration of large amounts of partially purified thyrotropic hormone to dogs failed to induce a diabetic condition.

6. The diabetogenic factor of the anterior pituitary gland is probably complex. The glycotropic and thyrotropic factors are possibly constituents of this complex, but prolactin is not.

I wish to thank Dr I. W. Rowlands for carrying out a number of the biological assays. This work was begun at University College, at which time I was in receipt of a grant from the Medical Research Council, which partly defrayed expenses, and a grant from the Diabetic Association, which provided for technical assistance. I wish to express my sincere thanks for these grants, and also for the valuable technical assistance of E. A. Woollett.

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# LXX. THE ACTION OF NITROUS ACID ON *p*-CRESOL AND TYROSINE

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In connexion with an investigation of the inactivation of pepsin by nitrous acid, the results of which appear in the next paper, we have been led to examine the action of nitrous acid on tyrosine. It seems to be generally supposed [Gibbs, 1926–27] that the yellow product obtained when nitrous acid acts on dilute aqueous tyrosine or *p*-cresol is an *o*-nitroso compound. We have now obtained evidence that although this compound is first formed, it does not accumulate, but at once undergoes further reaction. The usual product is chiefly a diazo compound accompanied by a nitro compound and photolysis products from the diazo compound (possibly also condensation products), the proportions varying with the conditions. We have also proved that substances belonging to the class of *o*-nitrosophenols are formed in the Millon reaction, but that they differ from the simple derivatives.

### *Reaction scheme for* p*-cresol and tyrosine*

The scheme in Fig. 1 summarizes the reactions which we have studied. It applies to both tyrosine and *p*-cresol, which differ only kinetically, apart from the deamination of the tyrosine; and it permits a wide choice of reactions according to conditions. The evidence for the scheme is based partly on conditions of formation and partly on the properties of the products, and is therefore diffused throughout the paper without being given separate consideration. Tyrosine and *p*-cresol will be referred to collectively as "phenols", since other monohydric phenols probably conform.

The chief possibilities are as follows:

(1) With dilute nitrite and phenols in acetate buffer plus $CuSO_4$ at temperatures from 0 to 40° the nitroso compounds are trapped as the very stable Cu derivatives.

(2) Under similar conditions in absence of $CuSO_4$ little or no nitroso compound can be detected at any stage of the reaction by adding $CuSO_4$ since it is rapidly transformed further. If, however, very dilute nitrite is added to a large excess of *p*-cresol, considerable amounts of nitroso compound are formed.

(3) The products of further reaction depend on the concentrations of $HNO_2$ and phenols, and on the temperature. If the ratio $[HNO_2]/[\text{phenol}]$ is low, red substances are obtained. Their structure and origin are at present unknown, but the product from *p*-cresol sometimes crystallizes directly from the reaction mixture. If the concentration of $HNO_2$ is increased and the concentration of phenol is not too high the chief product in the dark is diazo compound, unless air or nitrogen is bubbled through the solution. (Such treatment removes nitric oxide as explained below.) At still higher concentrations of nitrous acid, the yield of

diazo compound falls off, probably in favour of mono- or di-nitro compound. In the case of *p*-cresol these three regions are telescoped, while with tyrosine the middle one is much the most extensive.

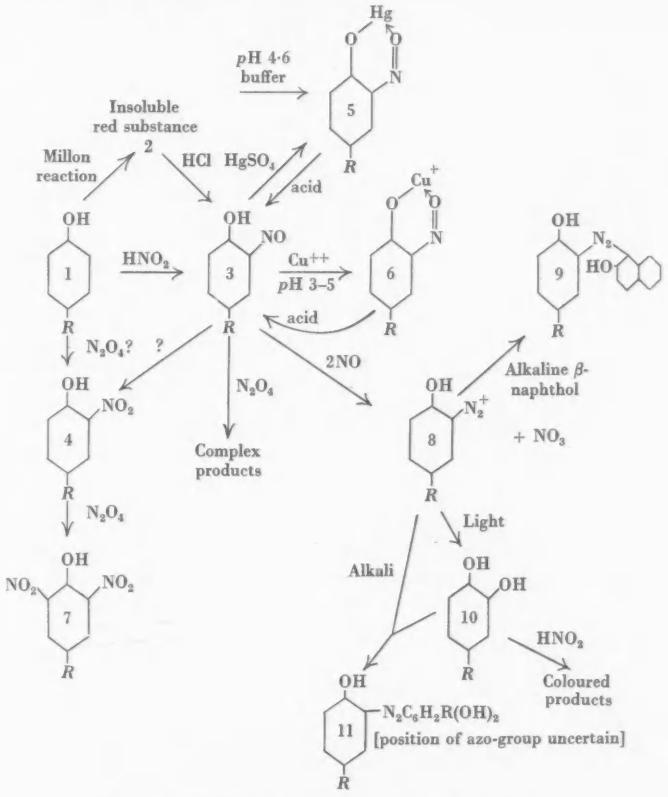


Fig. 1. $R = CH_3$, $CH_2.CH(NH_2)CO_2H$ or $CH_2.CHOH.CO_2H$.
Substances 3, 4, 6, 7, and 9 have been obtained crystalline.

*The decomposition equilibrium of nitrous acid*

The above conditions can be partly explained by consideration of the decomposition equilibrium of nitrous acid.

The reactions

$$4HNO_2 \overset{1}{\rightleftharpoons} 2N_2O_3 + 2H_2O \overset{2}{\rightleftharpoons} N_2O_4 + 2NO + 2H_2O \overset{3}{\rightleftharpoons} HNO_3 + HNO_2 + 2NO + H_2O$$

have been described by Abel *et al.* [1930].

The equilibrium point of reaction 3 normally lies well towards the right, but it is established much more slowly than the other two equilibria, which maintain the curious relationship:

$$\frac{[NO]^2\,[N_2O_4]}{[HNO_2]^4} = \text{const.}$$

*Explanation of above-mentioned conditions.* (1) Dilute $HNO_2$ is used to avoid the action of $N_2O_4$ on the phenols. Acetate buffer is better than mineral acids because it allows the $HNO_2$ to be continuously replenished from an internal reserve of $NO_2$. The *p*H of $HNO_2$ is about 3·3; with the concentrations of nitrite commonly used by us, e.g. $N/16$, the concentration of $HNO_2$ ranges from about 0·02 $N$ at *p*H 3·7 to about 0·002 $N$ at *p*H 4·7. A further reason for using this region of *p*H is that the Cu derivative is more stable than at lower *p*H values.

(2) The rate of formation of nitroso compound has been shown to be proportional to $[HNO_2] \times [\text{phenol}]$ by kinetic experiments which will be published later, while its further transformation into diazo compound will probably depend on $[\text{nitroso compound}] \times [NO]^2$. Hence low values of $[HNO_2]$ and high concentrations of phenols favour accumulation of nitroso compound.

(3) The unknown red substances are obtained whenever there is not enough NO or $NO_2$ or $Cu^{++}$ present to react with the nitroso compound as fast as it is formed. We think we have excluded the possibility of their formation from the nitroso compound, either by further reaction with the $HNO_2$ system, or by condensation with itself or anything else. (Autocondensation does occur, but quite slowly and to a different product.) We are almost driven to postulate a precursor of the nitroso compound, e.g. an addition product of the phenol and $HNO_2$, as postulated by Veibel [1930, 1, 2]. This precursor could undergo side reactions to give the red substance unless it were rapidly removed in other ways.

*Preparations and properties of products*

*Nitroso compounds.* The purple Cu chelated derivatives of the *o*-nitrosophenols are very stable between *p*H 3 and 8, particularly in presence of an excess of cupric salts. They are therefore formed under these conditions preferentially to any other derivatives. The similar derivative of phenol itself has been described by Baudisch [1918] though the preparation here described seems to be new. There are two derivatives from *p*-cresol. One, formed in presence of one equivalent of Cu is insoluble in water, but soluble in many organic solvents, while the other, formed in presence of excess Cu, is soluble in water and not extracted from aqueous solution by organic solvents. These are presumably the di- and mono-derivatives respectively; a supposition which agrees with the analysis of the supposed Cu di-derivative, given below. The corresponding derivatives from tyrosine are both water-soluble and not easily separated.

*Preparation of Cu di-*o*-nitroso-*p*-cresol, and* o*-nitroso-*p*-cresol*

10·8 g. *p*-cresol (0·1 mol.) were dissolved in 400 ml. of acetate buffer of *p*H 4·6, and 50 ml. of $M$ $CuSO_4$ were added. 59·3 ml. of 4·22 $N$ $NaNO_2$ were added and the solution made up to 1 l. and left 2 days at room temperature. Black crystals separated and were sucked off; the yield was 22 g. of damp cake. It was recrystallized by dissolving in hot alcohol and precipitating with water, or better, by evaporating a solution in a 2:3-alcohol:chloroform mixture. (Found: Cu 19·15%. $C_{14}H_{12}N_2O_4Cu$ requires Cu 18·95%.) The substance can conveniently be kept as an $M/10$ solution in the alcohol-chloroform mixture. It is also readily soluble in dioxane.

To obtain free *o*-nitroso-*p*-cresol for any experiment, the alcohol-chloroform solution may be shaken twice with $N$ $H_2SO_4$, to decompose the Cu complex, and washed twice with water, a chloroform solution of the nitroso compound resulting. The nitroso compound may be obtained solid by extraction of such a solution with a small amount of $N$ NaOH, and acidifying at 0°, when orange

crystals separate. They may be sublimed at 38°/20 mm. pressure, M.P. 58·5° (uncorr.). Evaporation of the chloroform solution gives nearly colourless crystals, but much is lost through volatilization.

*Properties.* The Cu di-*o*-nitroso-*p*-cresol is very stable, but decomposes explosively on heating. It is readily interconvertible with the water-soluble mono-derivative, according as nitroso-*p*-cresol or Cu is in excess.

It may be mentioned here that ordinary phenol also gives Cu *o*-nitrosophenols under conditions such as described above, whereas in ordinary nitrosation it gives very little *o*-derivative.

The free *o*-nitroso-*p*-cresol is fairly stable in dilute aqueous solution, but solutions stronger than $N/10$ apparently undergo condensation fairly rapidly in either water or organic solvents. It is acted on very rapidly by 50 % conc. HCl. A solution of the orange-red Na salt, made by shaking $N/10$ NaOH with an excess of the chloroform solution, is rather more stable, but seems to oxidize in the air in the course of some weeks. The compound gives the Liebermann nitroso reaction, and forms bright red nickel and mercuric derivatives. These properties, and the smell and volatility, agree with those of *o*-nitrosophenol as described by Baudisch [1918].

The purple colour of the Cu mononitroso-derivative may be used for its colorimetric estimation. Since the Cu solutions used are coloured green before any purple nitroso compound is present, monochromatic (sodium) light was used to avoid difficulties of colour matching. Colour intensities were measured in terms of glass filters as permanent standards. A solution was assigned a value of 100 when it matched a certain filter at a setting of 10 mm. The increase in colour units was taken as proportional to the amount of nitroso compound formed. By this means the yield of Cu *o*-nitroso-*p*-cresol was measured under various conditions, for which purpose a solution prepared by weighing out the solid di-derivative, and dissolving in an excess of buffered $CuSO_4$, thus forming the mono-derivative, was used as standard. The yield was found to be 95 % ± about 2 % in experiments at 38°, room temperature and 0°.

Experiments were also made to determine the effect of bubbling $N_2$ through the solution on the yield of the derivative from tyrosine. A solution was made up, at *p*H 3·7, containing 2 mg. tyrosine per ml., and $NaNO_2$ at a concentration of $N/8$. This was split into two parts, and each was heated at 38° for 6 hr., till the reaction was nearly complete, one being gassed with $N_2$ and the other not. Portions were removed at intervals, diluted 1 : 6 with buffer and their colour intensities determined. The final intensities were for the gassed solution 130 units, for the untreated 122 units; the slight excess in the former is attributable to evaporation. There is no significant difference; that is, neither NO nor $NO_2$ attacks the Cu compound with appreciable velocity, a conclusion which is supported by various qualitative experiments.

*Tryptophan* gives no coloured complex with $HNO_2$ in presence of Cu.

### *Nitrosophenols and the Millon reaction*

We have examined briefly the relation between the formation of *o*-nitrosophenols and the Millon reaction. *o*-Nitroso-*p*-cresol forms an intensely red mercuric derivative, at *p*H about 3–4, and may be prepared from *p*-cresol using Hg instead of Cu. This complex is not identical with the red substance obtained in the Millon reaction, since it is chloroform-soluble and instantly dissociated in $2N$ $H_2SO_4$. The red precipitate, obtained by performing the modified Millon reaction [Cole, 1933] on *p*-cresol, is not soluble in chloroform, and is fairly stable in $2N$ $H_2SO_4$ acid, but is converted by shaking with acetate buffer of *p*H 4·6 into

a chloroform-soluble substance, apparently identical with the mercuric complex of *o*-nitroso-*p*-cresol. On shaking the red chloroform solution with $2N$ $H_2SO_4$ even in presence of much $HgSO_4$, a yellow chloroform solution is obtained, which, when shaken with buffered $CuSO_4$ at *p*H 4·6, gives a purple aqueous layer; this behaviour is identical with that of the mercuric complex of *o*-nitroso-*p*-cresol.

If an excess of NaCl is added to the mercurial phenol obtained in the modified Millon test by heating *p*-cresol with a solution of $HgSO_4$ in $2N$ $H_2SO_4$, the solution so obtained fails to give the Millon test on addition of $NaNO_2$ as is well known. If not too much nitrite is added, a yellow substance is obtained soluble in water but not in chloroform, which gives a red colour with excess of $HgSO_4$, or a purple solution with $CuSO_4$, even in $2N$ $H_2SO_4$. It is therefore an *o*-nitrosophenol, though not *o*-nitroso-*p*-cresol itself, since the latter is soluble in chloroform and does not form complexes at so low a *p*H. If an excess of nitrite is used in the above experiment, diazo compound can be detected in the resulting yellow solution.

This behaviour can be explained on the assumption that the products insoluble in chloroform are mercurated in the nucleus, so that the red substance responsible for the Millon reaction would be a mercurated mercury complex of an *o*-nitrosophenol. The red substance obtained under similar conditions from tryptophan must be different in nature, and when it is decolored with NaCl, the product does not develop a colour with cupric salts.

Lugg [1937] has been led to postulate two classes of *o*-nitrosophenols, one of which gives red mercuric complexes, while the other, obtained from tyrosine and other phenols in absence of mercuric salts, does not. It is clear that the latter derivatives are what we have shown to be diazo compounds. There are, as we have shown above, two classes of nitroso compound, but both give red mercuric complexes under appropriate conditions.

The distinction drawn by Lugg between *o*-quinone monoximes and *o*-nitrosophenols seems now to be unnecessary; we have found no trace of such tautomerism when dealing with free *o*-nitroso-*p*-cresol, and the chelated metallic derivatives have a structure which is a resonance hybrid of the two types.

*Diazo compounds*

Diazo derivatives are formed with great ease by the action of $HNO_2$ on tyrosine and *p*-cresol. The one formed from tyrosine is much the more stable against further action of nitrous acid, and is formed under almost all conditions when tyrosine and $HNO_2$ react between 0 and 40°. Even if only one equivalent of $HNO_2$ is present, some diazo compound is formed from tyrosine, so that the reaction can compete with the deamination.

We have not isolated the diazo compounds, but they show characteristic colour reactions, by which they may be detected and estimated. They give red or purple coupling products in alkaline solution with such phenols as $\beta$-naphthol, resorcinol or phloroglucinol, and, if exposed to light in alkaline solution, form a red substance, which is photosensitive itself, so that the colour shows a maximum with time. This colour has been shown to be due to the coupling of unchanged diazo compound with a catechol derivative formed on irradiation by loss of nitrogen. Such a reaction of *o*-diazophenols has already been observed by Schmidt & Meier [1931]. If irradiated in sufficiently acid solution, these compounds give no colour, but if the irradiated solution is made alkaline, it will couple with fresh diazo compound. Though catechol does not readily couple, homocatechol, and more readily, "dopa" (3:4-dihydroxyphenylalanine) will couple, in alkaline solution, with diazodeaminotyrosine.

The diazo compounds do not couple with monohydric phenols, or with aniline, benzidine or dimethylaniline. They do, however, couple with α-naphthylamine, slowly in the cold, more rapidly on warming, at *p*H about 3.

The yield of diazo compound from tyrosine, in presence of various concentrations of $HNO_2$ has been investigated at 0 and 38°; a maximum is found at intermediate concentrations. The following figures give the amount of diazo compound formed in arbitrary units, measured colorimetrically by coupling with resorcinol, in presence of the respective concentrations of $NaNO_2$, at *p*H 3·7. One set of experiments at 0° was carried out with a layer of paraffin floated on the solution (as explained above this increases the NO concentration). The times before sampling were inversely proportional to the nitrite concentrations, so that an equal fraction of the reaction had occurred in each case (about 90%, as deduced from kinetic measurements).

| Final conc. $NaNO_2$ | 0° | 0° paraffin | 38° |
|---|---|---|---|
| 2*N* | 90 | — | 73 |
| *N* | 95 | 99 | 81 |
| *N*/2 | 97 | 99 | 83 |
| *N*/4 | 102 | 107 | 81 |
| *N*/8 | 100 | 104 | 65 |
| *N*/16 | 94 | 95 | 53 |
| *N*/32 | 85 | — | — |

The results indicate a definite optimal concentration of $HNO_2$.

The reaction mechanism by which diazo compounds are formed in this reaction has been determined. Kinetic data, to be given in detail in a later paper, indicate that the nitroso compound is a precursor of the diazo compound. It is known that "nitrous fumes" can convert other nitroso compounds into diazo compounds in ethereal solution, the reaction being

$$R.NO + 2NO \rightarrow R.N_2.NO_3.$$

We have shown that *o*-diazo compounds are formed from ethereal or chloroform solutions of the *o*-nitrosophenols in the same way, and the above-mentioned effect of bubbling $N_2$ supports the view that the reaction is the same in aqueous solution. Ethereal $N_2O_3$ or $NO_2$ give no *o*-diazo compounds.

These diazo compounds are extremely stable in absence of excess $HNO_2$; their solutions may be boiled without decomposition, and they do not undergo the Sandmeyer reaction even with HI except at 100° and then slowly. They react rather slowly with excess $HNO_2$, forming products whose nature has not been investigated. They may be reduced, presumably to amino or hydrazino derivatives, by the action of $TiCl_3$ or $Na_2S_2O_4$. A solution of diazopepsin titrated with $TiCl_3$ was found to require 3·8 atoms of H per diazo group corresponding with a reduction $R.N_2OH \rightarrow R.NH.NH_3 + H_2O$.

The coupling product of diazocresol and β-naphthol has been isolated. The preparation is as follows.

40 ml. of 0·2 *M* *p*-cresol in water (0·86 g.) and 10 ml. conc. HCl were made up to 1 litre with water, and 10 ml. 4 *N* $NaNO_2$ added. After 1 hr. the bright yellow solution was made strongly alkaline with 10 *N* NaOH, and 2 g. solid β-naphthol added. After 1 hr. more the solution was extracted with ethyl acetate, the extract washed with dil. NaOH to remove β-naphthol, and then twice with water, and the ethyl acetate dried with $CaCl_2$, filtered and evaporated on a water bath; the residual liquid was allowed to evaporate at room temperature; 1·3 g. of crystals

were obtained, this being a yield of 60 %. The product can be purified by sublimation at low pressure, or better by recrystallization from ethyl acetate; M.P. 109° (uncorr.). The substance is generally similar to the phenolazo-β-naphthol described by Niementowski [1902], though of a lower M.P. (phenolazo-β-naphthol melts at 193°). The yield of 60 % shows that, at least at one stage of the reaction between *p*-cresol and $HNO_2$, the amount of diazo compound present must be large.

*Nitro compounds and condensation products*

The study of the other products has been greatly hindered by lack of colour reactions for their detection and estimation. It was thought at first that the product obtained by the action of $HNO_2$ gassed with $N_2$ on *p*-cresol might be a nitro compound; but though the product of the reaction was extractable by ether, no definite compound could be isolated. By shaking a dilute ethereal solution of *p*-cresol with an aqueous mixture of acetate buffer and moderately concentrated $NaNO_2$ and evaporating the separated ether layer a good crop of crystals was obtained. These were recrystallized from ether plus light petroleum and proved to be dinitro-*p*-cresol, M.P. 81°. The same compound was got by the action of mixtures of $NaNO_2$ and 60–90 % conc. $HNO_3$, both from cresol and nitrosocresol. The mononitro compound, M.P. 33°, was obtained by the action of moderately concentrated nitrite and *p*H 3·7 buffer on very dilute *p*-cresol, 0·5 mg./ml., along with some diazo compound. We think, but cannot prove, that these are all cases of direct nitration by $N_2O_4$.

Various attempts were made to produce the nitro compound from the nitroso compound under gentle conditions in acid solution by oxidation, but without success. Titration of a solution of nitroso-*p*-cresol in *p*H 3·7 buffer with $KMnO_4$ showed that each mol. took up 6O, which presumably indicated the opening of the benzene ring.

*Application to pepsin.* Some, but not all, of the tyrosine groups in pepsin can be converted into diazo derivatives or Cu nitroso derivatives, without denaturing the protein or completely inactivating it. This will be described in the succeeding paper.

## Summary

1. The first recognizable product of the action of nitrous acid on monophenols is a nitrosophenol.

2. The *o*-nitrosophenol, however, rapidly undergoes further reaction, chiefly with nitric oxide to form the *o*-diazophenol.

3. The *o*-nitrosophenol can be trapped and isolated, via the very stable copper complex.

4. The red product of the Millon reaction is not the simple mercury-*o*-nitrosophenol, but is readily converted into it. The reverse change has not proved possible.

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*Note added March 1938.* The irradiation products of the diazophenols differ from "Dopa" and other catechol derivatives in that they couple much more rapidly with unchanged diazophenols at *p*H 10·4, and give less blue colour with Folin's "phenol" reagent at *p*H 5 and 100°, and practically no blue colour with arsenomolybdate at *p*H 3·7 and 100°. In these respects they resemble resorcinol very closely indeed. Attempts to obtain a fluorescein reaction with them were unsuccessful, possibly owing to insufficient purification; nevertheless it seems possible that they are 1:3- instead of 1:2-hydroxy derivatives.

# LXXI. THE ACTION OF NITROUS ACID ON PEPSIN

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In the search for the source of the specific activity of a protein such as pepsin one of the few available methods is to act on the protein with reagents which are known to attack certain types of group and to see whether the activity changes. Thus Herriott & Northrop [1934] and Herriott [1935–36] have acetylated pepsin with ketene, and Herriott [1937] has iodinated it. The conclusion from both researches was that tyrosine groups played a prominent part in the activity of pepsin.

The work here described was started some time ago when the conclusions would have been more novel. Nevertheless it provides further evidence of the participation of tyrosine in the activity of pepsin, and modifies the frequently quoted claim of Hugounenq & Loiseleur [1925] that the activity of pepsin is unaffected by nitrous acid. It also confirms other evidence that only a portion of the tyrosine groups in pepsin can react, at least at rates comparable with that of free tyrosine.

## *Preliminary experiments*

The earliest experiments were done in collaboration with Miss W. Hessey and Mr B. Mainwaring.

(1) *Action of $NaNO_2$ and HCl.* It was found that 1·7 *N* $NaNO_2$ plus 1·7 *N* HCl added to a solution of crystalline pepsin (about 1 %) at 25° caused rapid formation of a pale yellow precipitate. Activity determinations were uncertain owing to the insolubility of the precipitate, but very little activity could be detected. Hence the statement made by Hugounenq & Loiseleur [1925] that pepsin is not inactivated by nitrous acid "in a medium of hydrochloric acid" does not hold for pure pepsin though it may have held for the crude preparations available at that time.

(2) *Action of $NaNO_2$ in acetate buffer solutions.* It was found that provided that the *p*H was kept above 3·8 by acetate buffer no precipitate was formed, but the solution slowly became bright yellow and the activity fell off at a measurable rate to about half its original value, with a very much slower subsequent fall to zero. The yellow colour was accompanied by general absorption of light rising continuously from the blue to the short ultra-violet, and quantitative measurement showed that the band at 350–60 m$\mu$, due to $NO_2^-$, was not perceptibly diminished in intensity, while that at 275 m$\mu$, due chiefly to tyrosine groups in the pepsin, was diminished, although the total absorptions at both wavelengths were greatly increased by the yellow substance. This yellow substance is now known to be a diazo compound, since it shows all the colour reactions (coupling in alkaline solution with phenols, production of a red colour on irradiating in alkaline solution, colourless on irradiation in acid solution) characteristic of *o*-diazo-*p*-cresol and *o*-diazodeaminotyrosine, which we have

described in the preceding paper [1938]; some of these colour reactions have been used for its colorimetric estimation.

(3) *Liberation of $N_2$.* Over the *p*H range mentioned in the previous paragraph the concentration of undissociated $HNO_2$ was low, even in presence of *N* nitrite. Hence the decomposition to NO was slow enough to permit measurement of the rate of $N_2$ evolution from amino groups in a Barcroft or Warburg apparatus. The small amount of NO evolved was absorbed by permanganate dissolved in dilute phosphoric acid. It was essential to displace $O_2$ completely by purified $N_2$. The final technique was still scarcely quantitative, but it showed that under the conditions used most of the $N_2$ was liberated in half an hour, by which time the fall in activity and production of diazo compound had hardly started. This supports the contention of Hugounenq & Loiseleur that the peptic activity does not depend on the presence of amino groups, and it also agrees with the finding of Herriott & Northrop [1934] that the amino groups can be acetylated without change of activity. The experiments on $N_2$ evolution were done first in a Barcroft apparatus in collaboration with (Mrs) F. J. Philpot and later in a Warburg apparatus in collaboration with Dr E. Chain.

(4) *Action of $NaNO_2$ plus acetate buffer on amino-acids.* In presence of sodium nitrite at *p*H about 4 (the conditions finally chosen for pepsin) a yellow colour was given slowly by tyrosine, very much more rapidly by tryptophan, but not by cystine, leucine, aspartic acid, phenylalanine, histidine or arginine.

Of the amino-acids shown by Calvery *et al.* [1936] to be present in pepsin, the only ones not tried were lysine and glutamic acid, neither of which is likely to give a colour. The yellow product from pepsin gives the colour reactions of diazotyrosine as described in the preceding paper, and can therefore be considered as due to tyrosine, and possibly in part to tryptophan, though we have no definite evidence on the latter point.

## Final experiments

The preliminary experiments and the work of Herriott and Northrop on other reactions suggested that the partial inactivation of pepsin by $HNO_2$ was due to the action on tyrosine groups in the molecule. The final experiments were designed to prove this as rigidly as was practicable by showing not only that the kinetics of the partial inactivation of pepsin were identical with those of colour development from pepsin and free tyrosine, but that other amino-acids (tryptophan) and even other *p*-substituted phenols (*p*-cresol) react at quite different rates.

This is important for two reasons. First, even though tyrosine is concerned in the peptic activity of the protein, it is conceivable that this activity might depend on the presence of intact groups of both tyrosine and some other amino-acid, e.g. tryptophan. Since tryptophan is attacked by $HNO_2$ much faster than pepsin is inactivated, we conclude that it is not concerned in the activity of pepsin. Secondly, in order to exclude the possibility that the activity is due to some other *p*-substituted phenol, not yet isolated from pepsin, it is necessary to show that the velocity constants obtained are highly specific.

Runs were done with the following *p*-substituted phenols: *p*-cresol, *p*-hydroxyphenylacetic acid, tyrosol and α-bromopropionyltyrosine, to investigate the effect of the side chain on the reaction rate.

*Materials*

*Pepsin* was prepared by the following modification of the Northrop method: 100 g. Parke Davis 1 : 10,000 pepsin were dissolved in 100 ml. $H_2O$ and 100 ml. $N$ $H_2SO_4$ were added. The solution was filtered through Whatman No. 44 filter paper and 200 ml. saturated $MgSO_4$ were then dropped in from a capillary with mechanical stirring over a period of $\frac{1}{2}$ hr. The finely granular precipitate was centrifuged and redissolved in 70 ml. acetate buffer of *p*H 4·6 (AcONa, 0·5 $N$; AcOH, 0·548 $N$). The solution was dialysed with gentle stirring at 1 atm. excess pressure in cellophane, reinforced with netting, against a solution containing 0·3 $N$ NaCl, 0·0144 $N$ AcONa and 0·186 $N$ AcOH, *p*H about 3·5. Crystallization was complete after 5 days at room temperature, yield 18 g. of moist crystals. The product was recrystallized by dissolving in the *p*H 4·6 acetate buffer and repeating the dialysis: yield 60%.

The specimens used were generally twice crystallized. For storage the crystals were dissolved as before, pressure-dialysed against distilled water, and the concentrated nearly salt-free solution was left in a vacuum desiccator. The product consisted of brownish glassy flakes resembling the starting material in appearance. It was easily denatured by grinding, but otherwise would dissolve to a clear solution which possessed undiminished activity, was homogeneous in the ultracentrifuge, and as far as could be found contained no more non-protein material than the original solution of crystals. Great difficulty was experienced in determining the activity in absolute units, owing to variations in sensitivity in different preparations of haemoglobin.

As a result of earlier fruitless attempts to crystallize by dialysis it was observed that crystallization only occurred within a critical range of salt concentration, which was more restricted the lower the *p*H. Outside the critical range, only amorphous precipitates could be obtained. Below about *p*H 3·3 no salt concentration would yield crystals, while above *p*H 4·0 the pepsin became too soluble and crystallization was very slow and incomplete. When the salt concentration was at the limit of the critical range the edges of the crystals were rounded.

*The amino-acids and phenols* were the purest available, but no special precautions were taken as to their purity, since it was not likely to affect any of the conclusions of this paper.

*Technique*

Peptic activity was measured by the method of Anson & Mirsky [1932–33]. Control experiments showed that the method was unaffected by small amounts of $HNO_2$ added to the haemoglobin, and that the lower apparent activity of the pepsin derivative was not due to its instability under the conditions of the activity determination.

The light absorption of the yellow products was measured by means of a small Hilger spectrograph (not the standard model) used in conjunction with a rotating logarithmic sector and a Dobson microphotometer in a manner similar to that described by Dobson *et al.* [1926]; in experiments on tryptophan, and some on tyrosine, the increase in absorption at 411·5 m$\mu$, a wave-length at which neither unchanged amino-acid nor nitrite absorbs appreciably, was taken as a measure of the extent of reaction. This method was unfortunately the only one available for use with tryptophan.

In some experiments on pepsin and tyrosine the amount of the yellow diazo compound produced was estimated colorimetrically by one of the colour reactions mentioned in the preceding paper [1938], that with $\beta$-naphthol in alkaline

solution being found most convenient; although the colour produced with resorcinol was more intense, the excess resorcinol oxidized in air after some time, so that the intensity of colour was not constant. In a kinetic run, samples were taken out at intervals and made alkaline, sufficient alkali being added to give a final concentration of $N$ NaOH; finally 1 ml. of a 1% solution of $\beta$-naphthol in dilute alkali was added, and the colour estimated. The intensity of the red colour developed on illumination of the alkaline solution was also used to follow the reaction, but was much less satisfactory. All solutions of diazo compounds were kept in the dark.

The rate of formation of the Cu complex of the *o*-nitroso derivative from tyrosine (see previous paper) was also studied, the amount of the compound produced being estimated colorimetrically, using sodium light. The reaction was also followed in a very few instances by using Folin's reagent. The intensity of blue colour at the end of the reaction with tyrosine was about 10% of its initial value; the decrease in blue colour was taken as proportional to the extent of the reaction.

When the various methods had been shown to lead to the same velocity for tyrosine, and it had been established that they were measuring the velocity of the same reactions (i.e. $R.C_6H_4.OH + HNO_2 \rightarrow R.C_6H_3.(NO).OH + H_2O$, the rate-determining step which also controls the production of diazo compound) it was found most convenient to use the Cu nitroso compound, since it is the simplest, most convenient and most accurate.

The concentrations generally used for activity measurements were about 2·5mg. pepsin per ml., and samples were diluted 1:50 with distilled water for activity measurements. The concentration of tyrosine was about 1 mg./ml. for measurement as diazo compound or nitroso compound. When pepsin was used for these two latter methods, its concentration was chosen so that the amount of tyrosine reacting was about the same.

The final concentrations of $NaNO_2$ used were $N/16$ at 38° and $N$ at 0°. The two temperatures used, 38 and 0°, were obtained respectively by a gas thermostat, and by immersing in ice and distilled water kept in the cold room. Both were correct to 0·05°.

The *p*H was controlled by means of acetate buffers of ionic strength 0·5. Their composition was calculated from data given by Clark [1928]. Corrections were made for the buffering effect of the nitrite in calculating the concentration of free $HNO_2$. The buffering effect of the pepsin was negligible. The *p*H of buffers was occasionally checked by means of a glass electrode (the *p*H of 0·5 $N$ sodium acetate plus 0·548 $N$ acetic acid being taken as 4·6) and agreed within 0·05 unit. Systematic errors in *p*H were probably the limiting factor in the accuracy of the velocity constants finally obtained.

The concentration of free $HNO_2$ in equilibrium with nitrite at a given *p*H was calculated using a value for the dissociation constant extrapolated for 38° from the data of Klemenč & Hayek [1929] for 0, 12·5 and 30°. The values so obtained are given in Table I below.

Below *p*H 3·8 the product from pepsin precipitated even from quite dilute solutions, causing technical inconveniences, while above *p*H 5 the rate of alkaline inactivation of pepsin was comparable with that of the reaction under investigation. The final experiments were therefore confined to *p*H 3·8–4·6. Control experiments showed that within this range the inactivation of pepsin in absence of nitrite was negligible during the time of the experiment.

Table I. *Concentrations of $HNO_2$ in buffer solutions*

$NaNO_2 = 0{\cdot}0625$, Na acetate $= 0{\cdot}25$, 38°

| Buffer *p*H | Final *p*H | $[HNO_2]$ |
|---|---|---|
| 3·7 | 3·727 | 0·0137 |
| 3·75 | 3·775 | 0·0126 |
| 3·8 | 3·823 | 0·0116 |
| 3·9 | 3·920 | 0·00965 |
| 4·0 | 4·017 | 0·00810 |
| 4·1 | 4·115 | 0·00689 |
| 4·2 | 4·213 | 0·00567 |
| 4·3 | 4·311 | 0·00462 |
| 4·4 | 4·410 | 0·00372 |
| 4·5 | 4·509 | 0·00299 |
| 4·6 | 4·608 | 0·00239 |
| 5·0 | 5·004 | 0·000970 |

"Buffer *p*H" is the *p*H of the buffer at 18° before addition of nitrite.
"Final *p*H" is the *p*H at 18° after addition of nitrite, calculated allowing for the buffering effect of the nitrite.
$[HNO_2]$ is the concentration of free nitrous acid at 38 or 0° respectively.

$NaNO_2 = 1{\cdot}0$, Na acetate $= 0{\cdot}25$, 0°

| | | |
|---|---|---|
| 3·7 | 4·002 | 0·198 |
| 3·8 | 4·079 | 0·171 |
| 3·9 | 4·158 | 0·147 |
| 4·0 | 4·236 | 0·127 |
| 4·2 | 4·397 | 0·0944 |

Table II. *Velocity constants at 38°*

(*a*) $NaNO_2 = 0{\cdot}0625$; ionic strength $= 0{\cdot}313$

Method A. As diazo compound by photo-reaction.
,, B. As diazo compound by coupling with phenols.
,, C. As nitroso compound in presence of copper.
,, D. By decrease in blue colour with Folin's reagent.
,, P. By peptic activity. Method S. Spectrographically.

| Substance | Method | *p*H | $K_u$ | $K_b$ |
|---|---|---|---|---|
| Pepsin | P | 3·8 | 0·00226 | 0·00748 |
| | P | 3·8 | 0·00248 | 0·00821 |
| | P | 3·8 | 0·00246 | 0·00815* |
| | P | 4·0 | 0·00190 | 0·00875 |
| | P | 4·0 | 0·00186 | 0·00855† |
| | B | 4·1 | 0·00161 | 0·00895 |
| | P | 4·1 | 0·00160 | 0·00890 |
| | P | 4·2 | 0·00120 | 0·00815 |
| | A | 4·2 | 0·00119 | 0·00809 |
| | P | 4·2 | 0·00120 | 0·00815 |
| | P | 4·3 | 0·00098 | 0·00815 |
| | P | 4·3 | 0·00100 | 0·00823 |
| | P | 4·4 | 0·00078 | 0·00808 |
| | P | 4·5 | 0·00073 | 0·00936 |
| | P | 4·5 | 0·00067 | 0·00860 |
| | | | Mean | 0·00839 |
| Tyrosine | A | 3·8 | 0·00270 | 0·00895 |
| | C | 3·8 | 0·00285 | 0·00943 |
| | D | 3·8 | 0·00223 | 0·00739 |
| | C | 3·8 | 0·00228 | 0·00755 |
| | B | 4·0 | 0·00173 | 0·00796 |
| | C | 4·0 | 0·00178 | 0·00821 |
| | C | 4·0 | 0·00178 | 0·00821 |
| | A | 4·2 | 0·00115 | 0·00780 |
| | B | 4·2 | 0·00126 | 0·00855 |
| | B | 4·2 | 0·00120 | 0·00815 |
| | B | 4·2 | 0·00115 | 0·00780 |
| | C | 4·2 | 0·00119 | 0·00807 |
| | D | 4·2 | 0·00115 | 0·00780 |
| | C | 4·4 | 0·00076 | 0·00792 |
| | C | 4·6 | 0·00050 | 0·00805 |
| | D | 4·6 | 0·00051 | 0·00820 |
| | C | 3·8 | 0·00251 | 0·00832 |
| | C | 3·9 | 0·00212 | 0·00844 |
| | C | 4·0 | 0·00178 | 0·00821 |
| | C | 4·1 | 0·00147 | 0·00816 |
| | | | Mean | 0·00816 |

Table II (*cont.*)

| Substance | Method | *p*H | $K_u$ | $K_b$ |
|---|---|---|---|---|
| α-Bromopropionyl-tyrosine | C | 3·75 | 0·00260 | 0·00780 |
| | C | 3·8 | 0·00270 | 0·00895 |
| | | | Mean | 0·00838 |
| Tyrosol | C | 4·0 | 0·00285 | 0·0131 |
| | S | 4·2 | 0·00150 | 0·0115 |
| | S | 4·6 | 0·00070 | 0·0113 |
| | | | Mean | 0·0120 |
| *p*-Cresol | C | 3·75 | 0·00625 | 0·0190 |
| | C | 3·8 | 0·00540 | 0·0179 |
| | C | 4·0 | 0·00416 | 0·0191 |
| | | | Mean | 0·0187 |
| *p*-Hydroxyphenyl-acetic acid | C | 3·8 | 0·00268 | 0·00890 |
| | C | 3·9 | 0·00222 | 0·00885 |
| | C | 4·0 | 0·00202 | 0·00925 |
| | C | 4·1 | 0·00164 | 0·00912 |
| | | | Mean | 0·00903 |
| Tryptophan | S | 4·2 | 0·019 | 0·15‡ |

* Illuminated Hg vapour lamp. † In presence Cu; final activity = 30 %.
‡ Only approx.

(*b*) Effect of varying salt concentration. Tyrosine, *p*H 4·4, $NaNO_2$ = 0·0625, copper nitroso method

| Ionic strength $\mu$ | $K_u$ | Salt added |
|---|---|---|
| 0·425 | 0·0082 | $CuSO_4$ |
| 0·81 | 0·0085 | $Na_2SO_4 + CuSO_4$ |
| 0·875 | 0·0085 | $NaCl + CuSO_4$ |
| 1·31 | 0·00875 | $NaCl + CuSO_4$ |

## Table III. *Velocity constants at 0°*

(*a*) $NaNO_2$ = 1·0; total salt concentration = 1·25

| Substance | Method | *p*H | $K_u$ | $K_b$ | Remarks |
|---|---|---|---|---|---|
| Pepsin | P | 3·7 | 0·0077 | 0·00150 | |
| | B | — | 0·0069 | 0·00134 | |
| | P | 3·8 | 0·0045 | 0·00101 | |
| | | | Mean | 0·00128 | Methods as in Table I |
| Tyrosine | B | 3·7 | 0·0066 | 0·00128 | |
| | C | — | 0·0065 | 0·00126 | |
| | C | 3·8 | 0·0054 | 0·00121 | |
| | | | Mean | 0·00125 | |

(*b*) Effect of varying nitrite. Tyrosine, *p*H 3·8, method C

| Nitrite | Ionic strength | $K_u$ | $K_u'$ $\mu = 1·25$ | $[HNO_2]$ | $K_b$ |
|---|---|---|---|---|---|
| 0·5 | 0·75 | 0·0025 | 0·0026 | 0·103 | 0·00097 |
| 1·0 | 1·25 | 0·0054 | 0·0054 | 0·171 | 0·00121 |
| 1·5 | 1·75 | 0·0086 | 0·0083 | 0·225 | 0·00142 |

$K_u'$ is $K_u$ corrected for salt effect, assumed the same as at 38°.
If $V = k_{b_0}$ (tyrosine) $(HNO_2)$ $(1 + a'[NO_2^-])$, $a = 0·66$ and $K_{b_0} = 0·000775$.
Mean velocity of pepsin and tyrosine at 0°, corrected for salt effect and nitrite effect, i.e. calculated for $\mu = 0·3125$ and total nitrite = 0·0625 as used at 38°, is 0·00078.

## Results

(1) Under the conditions used, there being a very large excess of nitrite, all the reactions are pseudo-unimolecular. This is illustrated by the logarithmic plots for four runs in Fig. 1. A curve of peptic activity against time is also given, to illustrate that the final activity is 50%, the mean value found for the series being $50 \pm 1\%$. This final activity is fairly stable, but further diminishes at a

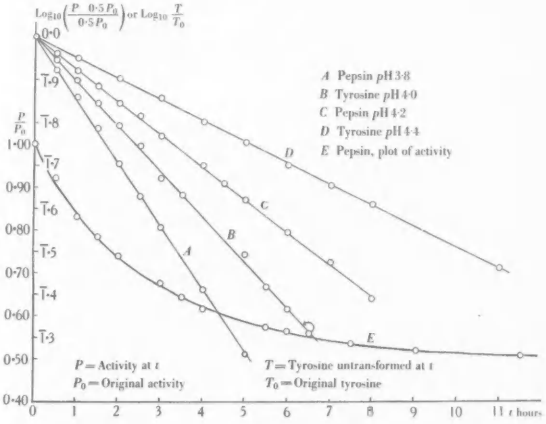


Fig. 1.

very low rate, which is comparable with the rate at which $HNO_2$ attacks diazo compound. If the $HNO_2$ is removed with urea or by dialysis no further loss of activity occurs.

(2) The pseudo-unimolecular constants for the inactivation of pepsin, for the production of diazo compound from tyrosine or pepsin, and the production of copper nitroso compound from tyrosine or α-bromopropionyltyrosine are almost identical under similar conditions, and with a constant $[NO_2^-]$ are proportional to the $[HNO_2]$.

*p*-Cresol and tyrosol react at quite different rates from pepsin or tyrosine, but the rate of *p*-hydroxyphenylacetic acid is not very different. However, application of the "Student" *t*-test to the rates of pepsin and *p*-hydroxyphenylacetic acid shows that the probability that the figures obtained are all members of the same set is only 0·02. To eliminate the possibility that differences in technique might upset this conclusion, four runs (the four last of the tyrosine series in Table II) were done by the same method which was used for *p*-hydroxyphenylacetic acid (the copper method), two before and two after the set of experiments on the latter substance, and they agreed excellently with earlier results. By application of the *t*-test, it was shown that the probability of the rates of tyrosine and *p*-hydroxyphenylacetic acid being members of the same set is only 0·01.

The rates of reaction of different *p*-substituted phenols are therefore sufficiently specific for conclusions to be drawn from them. There is, however, one factor which is difficult to eliminate, that is the influence of the formation of peptide or similar linkages on the rate of reaction. Two runs were done with

α-bromopropionyltyrosine, and the rates were found to agree very well with the rate of reaction of tyrosine. It is perhaps rather surprising, in view of the effect of different side chains on the rate of reaction, that the groups

$$—CH_2.CHOH.CO_2H \quad \text{and} \quad —CH_2.\underset{\displaystyle CO_2H}{\underset{|}{CH}}.NH.OC.CHBr.CH_3$$

in deaminated tyrosine and α-bromopropionyltyrosine respectively should have the same effect; but it appears from this that peptide formation does not appreciably influence the rate of reaction of the benzene nucleus. A further rather surprising fact is that the velocity constants of deaminated tyrosine and *p*-hydroxyphenylacetic acid do not appear to vary with *p*H, though the *p*H of the experiments is in the region of their *p*K, and hence ionization of side chains can have little effect.

Exposure to light during the reaction does not alter the reaction rate, as is shown by the run quoted and one or two others.

In Table II $K_u = \frac{-d \log_{10} (\text{activity or tyrosine})}{dt_{\text{min.}}}$, $K_b = \frac{K_u}{[HNO_2]} \times 0{\cdot}0384$, and is the apparent bi-molecular constant in litres/mol. sec.; $K_b$ is not, however, independent of nitrite concentration, as Table III (*b*) shows, but is proportional to $(1+a[NO_2^-])$. This may be interpreted with the aid of the reaction complex of phenol and $HNO_2$ assumed by Veibel [1930] to be in equilibrium with its components. The concentration of this complex will be proportional to [tyrosine] × $[HNO_2]$, its rate of breakdown to *o*-nitroso compounds gives the independent term and a reaction with $NO_2^-$ to give the same product gives the term proportional to $NO_2^-$. This effect of $NO_2^-$ is perhaps analogous to that found by Taylor [1929] in the reaction between $HNO_2$ and various amines. The salt effect is very small, which is consistent with the view that one of the species reacting is uncharged, e.g. $HNO_2$.

The identity of the rates of production of copper nitroso compound and diazo compound from tyrosine support the views put forward in our previous paper that the nitroso compound is a precursor of the diazo compound and is rapidly transformed into it.

(3) Tryptophan reacts nearly 20 times as fast as tyrosine. Several runs were done, but in most cases no good value could be obtained for the velocity constant, owing to the technical difficulties of the spectrographic method. One figure is quoted, to give the order of magnitude of the velocity constant.

(4) Table III gives velocity constants for pepsin and tyrosine at 0°. The substantial identity of the velocity constants at this temperature also very much increases the likelihood that the two reactions are really the same. Since the kinetics are not quite simple, the value of 10,400 calories which is calculated for the heat of activation for both these reactions depends on the exact magnitude of several correction factors applied to the velocity constant at 0°, and must be regarded only as giving the order of magnitude; moreover, the mechanism may, as suggested above to account for the effect of $NO_2^-$, be complex. However, the abnormally low value of the temperature coefficient is of interest, as it can be explained by the greater dissociation at the higher temperature of the intermediate complex mentioned above.

(5) The properties of the product from pepsin are being further investigated and will be described in a later paper. It has been shown to be homogeneous in the ultra-centrifuge if prepared at 0° and to have approximately the same sedimentation constant as pepsin, which supports the conclusion derived from the kinetic data, that the diminution in peptic activity is due to a specific

effect of the introduction of substituents into the tyrosine group rather than to general damage of the molecule.

The activity of the diazo compound is not changed when it is acted on by light, either in presence or in the absence of $HNO_2$. Reduction with $Na_2S_2O_4$ does not change the activity by more than 5%. Hence the diazo, hydroxy and several other derivatives have equal peptic activities.

(6) The purple colour developed from pepsin in presence of Cu and $HNO_2$ is approximately 45% of that expected from its tyrosine content. If the pepsin is broken down by autolysis at *p*H 2 and 38°, or hydrolysis with strong alkali at 100°, it then develops the same amount of colour as a tyrosine solution of equivalent concentration. This suggests that only a portion of the tyrosine in native pepsin is free to react, and recalls the observation of Herriott [1935–36] that several proteins, including pepsin, only give about 60% of the theoretical colour intensity with Folin's reagent at *p*H 8.

## Discussion

The chief result of the present work is to add nitrous acid to the brief list of reagents which can act in a known manner on a native protein without damaging the molecule as a whole. A more special result is to provide further evidence in support of the claim of Northrop and Herriott that tyrosine groups, or at least some phenolic groups, play a part in peptic activity. While Herriott's isolation of diiodotyrosine from iodinated pepsin showed conclusively that tyrosine groups in pepsin react with iodine, it did not exclude the possibility that the fall in peptic activity was due to one or two other phenolic groups reacting at the same rate as tyrosine. Our finding that *p*-cresol, tyrosol and *p*-hydroxyphenylacetic acid react at measurably different rates from tyrosine makes it increasingly probable that the phenolic group concerned with peptic activity is that of tyrosine itself. The very rapid reaction of tryptophan also excludes the possibility that unsubstituted tryptophan groups are essential for peptic activity.

The action of nitrous acid on pepsin seems to be simpler kinetically, as far as peptic activity is concerned, than that of either ketene or iodine. The unimolecular approach to a 50% active product, with only a very slow subsequent change, is unprecedented. That the 50% active product also possesses about 50% of its tyrosine groups intact is probably mere coincidence; but it is possible that those tyrosine groups which are protected from nitrous acid are nevertheless somehow able to participate in peptic activity.

In any case it is probable that those groups which do react with nitrous acid become quite inactive enzymically, since they can undergo subsequent changes, e.g. by irradiation, without causing any change in total peptic activity. The relationship between our 50% active product and the several products yielded by ketene and iodine is obscure, and can only be elucidated by further work, e.g. by successive action of the different reagents.

## Summary

1. The reaction between pepsin and nitrous acid has been studied up to *p*H 4·5 and at 0 and 38°.

2. The rate is proportional to the concentration of unchanged pepsin and of undissociated nitrous acid, and depends slightly upon the nitrite concentration.

3. The product is a yellow photosensitive diazo compound and has 50% of the original activity. Subsequent changes are very slow.

4. Comparisons with the action of nitrous acid on amino-acids and other substances support the view that the fall in activity is due to an action on the tyrosine groups in pepsin.

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# LXXII. OBSERVATIONS ON THE CRYSTALLIZABLE ALBUMIN FRACTION OF HORSE SERUM

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THE advantage of sodium sulphate as a reagent for use in the precipitation and crystallization of proteins has been pointed out in a previous paper [Kekwick & Cannan, 1936] and it was considered of value to attempt to extend this procedure to the serum proteins, and in particular to the crystallization of the albumin fraction. Extensive investigations on the fractionation of serum proteins with sodium sulphate have been made by Howe [1921], but in no case was a crystalline product prepared.

It soon appeared that the crystalline albumin fraction of horse serum was probably not homogeneous, a fact which has been observed a number of times, and most extensively investigated by Sørensen [1930].

Two crystalline fractions have been prepared and evidence with regard to their chemical individualities is presented, together with a survey of some of their physico-chemical characteristics.

## *The fractionation of horse serum proteins with sodium sulphate*

### *Globulin precipitation.*

In Howe's [1921] investigations diluted serum was always used and Homer [1919] stated that in undiluted serum sharp separation of the protein fractions did not occur either with sodium sulphate or other precipitating salts.

The avoidance of large dilutions is an obvious advantage for large scale preparations and consequently the precipitation curve of undiluted serum with sodium sulphate was reinvestigated.

Horse serum for this purpose, and for all subsequent preparations described in the paper, was obtained from healthy horses by collecting blood in a receptacle in the bottom of which had been poured sufficient 3 % sodium oxalate solution to make the final concentration 0·1 % when the jar was filled with blood. This concentration of sodium oxalate did not inhibit clotting, but a very loose clot was formed which shrank to about one-third of its initial volume in less than 12 hr. in the cold. Practically no haemolysis occurred during this period. After siphoning off the supernatant serum and centrifuging lightly to remove suspended particles a water-clear pale yellow serum was always obtained. Such serum occasionally contained minimal quantities of fibrinogen.

The precipitation curve was investigated at 30°, 50 ml. samples of a batch of serum being added to weighed quantities of anhydrous sodium sulphate, which was dissolved by the aid of mechanical stirring. After 2 days' standing the precipitates were filtered off and washed with 25 ml. sodium sulphate solution of the concentration used for the precipitation. The filtrate and washings were combined and made up to volume and the N content determined directly by the micro-Kjeldahl method.

In Fig. 1 the filtrate N per 100 ml. serum is plotted against sodium sulphate concentration. A distinct plateau appears in the curve extending from 19 to 21 g. $Na_2SO_4$ per 100 ml. The use of 20 g. $Na_2SO_4$ per 100 ml. serum for the precipitation of the globulins was therefore adopted for undiluted serum.

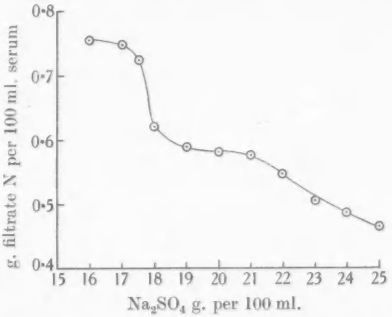


Fig. 1. The precipitation curve of horse serum with sodium sulphate. $T = 30°$.

Howe [1921], using serum diluted 1 : 10, found breaks in the precipitation curve from 13·5 to 14·5, 16·4 to 17·4 and 21 to 22 g. $Na_2SO_4$ per 100 ml. He recommended the use of 21·5 g. $Na_2SO_4$ per 100 ml. for globulin precipitation under these conditions. For undiluted serum a second discontinuity is suggested by the present data around 16 g. $Na_2SO_4$ per 100 ml. but this was not adequately investigated.

*Crystallization and fractionation of albumin fraction*

As a general rule the serum was worked up in 4 l. batches, all the procedures being carried out in a warm room at 30° unless otherwise stated.

The globulins were precipitated by addition of 20 g. $Na_2SO_4$ per 100 ml. serum and allowed to stand overnight before filtration. The albumin filtrate was then acidified with 0·2 *N* $H_2SO_4$, the usual precautions being observed (continuous mechanical stirring; delivery of the acid from a small orifice below the surface of the liquid) until an apparent *p*H of 4·8 to 4·9 was attained.

This usually required 230 ml. acid per l. of filtrate. The *p*H was controlled externally by the use of suitable phthalate buffers [Clark, 1928] with bromocresol green as an indicator, allowance being made for the colour of the serum and for the effect of the $Na_2SO_4$ present. The *p*H is advisedly referred to as "apparent", since the protein error of the indicator is considerable, and larger with serum albumin than figures in the literature indicate.

Crystallization usually commenced as the final amounts of acid were stirred in; then for every 100 ml. of acid added 20 g. of anhydrous $Na_2SO_4$ were stirred in and crystallization was allowed to come to completion by standing for 24 hr.

After standing, the crystals were filtered off and allowed to drain thoroughly. In order to ascertain that crystallization had been complete the filtrate was on many occasions tested out for further crystallization; it was found that either a slight amorphous precipitate formed, or if there were any crystals the amount was negligible.

The carefully drained crystals were then extracted with a volume of distilled water at 30° equal to one-tenth that of the initial serum, the extraction being continued for 16 hr. with occasional stirring. The undissolved material, mostly

crystalline but showing some amorphous substance was then filtered off, drained and extracted for several days at 2° with a quantity of distilled water equal to that used in the first extraction, after which the green amorphous residue was filtered off and discarded.

The filtrate from the first extraction will be referred to as fraction A, that from the second as fraction B, and the green material as "insoluble residue". The data given in Table I show the average quantities of these materials obtained by such a fractionation procedure. The actual amounts vary with the different samples of serum, and the yield of crystalline material tends to fall if the serum is kept.

Table I

| | g. $N_2$/l. serum |
|---|---|
| Total albumin fraction | 3·7 |
| Total crystalline albumin | 2·5 |
| Fraction A | 0·6 |
| Fraction B | 0·9 |
| "Insoluble residue" | 1·0 |

*Recrystallization and the carbohydrate-nitrogen ratio of the fractions*

The fact that crystalline serum albumin can be separated into fractions of different carbohydrate contents was first shown by Sørensen [1930] and Sørensen & Haugaard [1933]. Recently Hewitt [1936; 1937] has reinvestigated this and other aspects of the problem.

It was considered that an investigation of the carbohydrate-nitrogen ratio of the fractions A and B, particularly from the point of view of its variation during subsequent recrystallizations, would give some evidence as to the chemical homogeneity of the fractions.

The recrystallizations were carried out at 30° and conditions were sought such that at each recrystallization between 85 and 95 % of the fractions was recovered. For fraction A this was achieved by recrystallization from solutions containing 3·0 g. protein per 100 ml., the sodium sulphate concentration being brought up to 20 g. per 100 ml. For fraction B the corresponding conditions were 4·5 g. protein per 100 ml. and 15 g. $Na_2SO_4$ per 100 ml. The *p*H was carefully maintained at 4·8–4·9 as in the initial crystallization procedure. The protein concentrations were determined by micro-Kjeldahl, an arbitrary factor of 6·5 being adopted for the conversion of nitrogen to protein for both fractions.

Samples of crystallized material and mother liquors were removed at each recrystallization and dialysed in cellophane against toluene-saturated distilled water at 0° until the dialysate showed no $SO_4''$ test after 16 hr. contact with the solution. In some cases the mother liquors were concentrated by pressure dialysis to obtain a suitable protein concentration. The carbohydrate-nitrogen ratio was then determined for each sample, the nitrogen by the micro-Kjeldahl method and the carbohydrate by the Sørensen & Haugaard [1933] method. Details of the procedures are given below.

Table II. *Carbohydrate-nitrogen ratios for crystallizations of fractions A and B*

| | Fraction A | | Fraction B | |
|---|---|---|---|---|
| Crystallization no. | Crystals | Mother liquor | Crystals | Mother liquor |
| 1 | 0·204 | — | 0·022 | 0·204 |
| 2 | 0·123 | 0·25 | 0·019 | 0·179 |
| 3 | 0·141 | 0·111 | 0·0069 | 0·048 |
| 4 | 0·123 | 0·104 | 0·0052 | 0·023 |
| 5 | 0·136 | 0·038 | 0·0055 | 0·0079 |

In Table II the results of one investigation of this type are given for both fractions A and B, the ratios having been calculated as mg. carbohydrate per mg. nitrogen. Similar data, not included here, have been obtained from further experiments which confirm the results in Table II.

Considering fraction A it will be observed that its ratio remains substantially constant from crystallization 2 onwards after a material very rich in carbohydrate was eliminated by crystallization 2. The mother liquors from crystallizations 3 and 4 approximate in ratio to the crystals though that from crystallization 5 shows an unusual discrepancy.

For fraction B the ratio is of a different order of magnitude and becomes sensibly constant after the second crystallization. The data for the mother liquors shows the elimination of a substance considerably richer in carbohydrate than the crystals, but when the fifth crystallization is reached this material has been practically completely removed.

On the basis of these data it is suggested that the method of fractionation described results in the separation of two chemically distinct crystallizable substances.

It may be emphasized here that to adduce evidence proving conclusively that a protein is chemically homogeneous is a matter of the utmost difficulty.

The recrystallizations were also controlled by sedimentation velocity measurements (for methods see p. 556). These showed the presence in fraction A, during early crystallizations, of a very slight amount of heavily sedimenting material, probably globulin, which was however completely removed after four crystallizations. A twice crystallized sample of fraction B showed no contamination.

In general appearance the two fractions are rather alike, the crystals being of similar needle-shaped form, but those of fraction A were usually smaller than those of fraction B. Fraction A was however pigmented, a 2% solution being a bright yellow colour, whereas a corresponding solution of fraction B was almost colourless.

The crystal precipitate removed by filtration can be rapidly reduced to a dry powder by circulating air at 25° over it. The product can easily be ground to a fine powder and is completely redissolved on treatment with water, from which solution it can be recrystallized. A sample of such material which had been kept at room temperature for 3 years retained its ability to be crystallized. Ultra-centrifugal investigation showed that this treatment caused the protein to become slightly polydisperse, but for many purposes this is not a very serious disadvantage. None of the preparations used in the investigations in this paper had been dried.

*The nitrogen and carbohydrate content of the fractions*

A determination of the percentage of nitrogen in each of the fractionated materials, in addition to being of interest itself, enables an estimation of the percentage of carbohydrate to be made when taken in conjunction with the ratios of Table II.

Samples of five times crystallized material were dialysed as before. On aliquots of the solution nitrogen was determined by micro-Kjeldahl, and the amount of protein by drying to constant weight at 105° followed by ashing in an electric muffle at 450°.

The nitrogen values found, together with carbohydrate contents calculated by combination with the data of Table II for the five times crystallized material, are given in Table III, on an ash-free basis.

Table III. *Nitrogen and carbohydrate content of fractions A and B (five times crystallized)*

| | % N | % carbohydrate | Ash (% dry wt.) |
|---|---|---|---|
| Fraction A | 14·35 | 1·95 | 0·38 |
| Fraction B | 15·16 | 0·083 | 0·23 |

## Experimental

(1) *Determination of nitrogen.*

Estimations were carried out in triplicate by the micro-Kjeldahl method, suitable blank determinations being made concurrently. Selenium oxychloride was used as catalyst.

The method was tested on a sample of crystallized egg albumin in parallel with the ordinary copper sulphate method and identical results were obtained. With the selenium catalyst, however, the digestion time was considerably shortened.

(2) *Estimation of carbohydrate.*

Estimations of carbohydrate were carried out by the method of Sørensen & Haugaard [1933]. Into 15 ml. 60 % $H_2SO_4$ were pipetted 1 ml. protein solution and 2 ml. 2 % orcinol in 60 % $H_2SO_4$, a blank omitting the orcinol being set up for each protein solution. After thorough mixing the solutions were heated in a water bath at $80 \pm 1°$ for 20 min., plunged into ice water and cooled in the dark. The extinctions were measured in a Zeiss step photometer[1] using the filters S 43 and S 53.

Calibration curves were made using a mixture of equal quantities of glucose and mannose, the carbohydrate content of the protein solutions being read off from the curve.

More weight was given to the extinctions with the S 43 filter, those from the S 53 filter being used for corroboration of the general magnitude of the carbohydrate content of the protein solutions.

The use of a glucose-mannose solution for reference was arbitrary, comparative data being the chief objective.

### *Some physico-chemical characteristics of the fractions*

Having established a chemical differentiation, it was considered of interest to determine whether the fractions showed any divergence in some of the more common physico-chemical characteristics usually determined for proteins. For this purpose measurements of sedimentation velocity, electrophoretic velocity and diffusion were made on both fractions. For these measurements a six times crystallized fraction A and a three times crystallized fraction B were used.

*Sedimentation velocity measurements.*

Sedimentation velocity measurements were made using the light adsorption method, a mercury vapour lamp in conjunction with chlorine and bromine filters ($\lambda = 250$–$270 m\mu$) being used as a light source [Svedberg, 1925; Svedberg & Eriksson, 1932]. The centrifugal field was approximately 200,000 times gravity in all experiments.

The sedimentation constants corrected to pure water at 20° together with other relevant data are given in Table IV. Sufficient electrolyte was present in the solutions to suppress any charge effect on the sedimentation.

[1] I am indebted to Prof. C. R. Harington for allowing me to use this apparatus.

Table IV. *Sedimentation constants of fractions A and B*

| Buffer | Total salt conc. ($M$) | $p$H | $S_{20} \times 10^{13}$ Fraction A | $S_{20} \times 10^{13}$ Fraction B |
|---|---|---|---|---|
| HOAc, NaOAc, NaCl | 0·2800 | 3·74 | (4·10) | (4·31) |
| HOAc, NaOAc, NaCl | 0·3000 | 5·04 | 4·34 | 4·52 |
| $KH_2PO_4$, $Na_2HPO_4$, NaCl | 0·2800 | 6·63 | 4·52 | 4·55 |
| $KH_2PO_4$, $Na_2B_4O_7$, NaCl | 0·2500 | 8·54 | 4·46 | 4·36 |
| | | Mean | 4·44 | 4·48 |

The experiments cover almost completely the stability range given for serum albumin, viz. $p$H 4–9 [Svedberg & Sjögren, 1930]; in fact the first pair are just outside this range. The mean values for each fraction, excluding the first pair of results, are in excellent agreement with the established value for the sedimentation constant for horse serum albumin, viz. $S_{20} = 4{\cdot}5 \times 10^{-13}$ [v. Mutzenbecher, 1933], and the difference between them is not significant. No sign of heterogeneity was shown by the absorption curves of either fraction.

*Measurements of electrophoretic mobility.*

Using the method of Tiselius [1930] a series of measurements of the electrophoretic mobilities of the fractions was made, with the same recording conditions as for the sedimentation velocity experiments. Acetate buffers (0·02 $M$ NaOAc + $x M$ HOAc) were used throughout, the region studied being in the vicinity of the isoelectric point alone. All experiments were conducted at 20°. The data obtained are given in Table V and Fig. 2.

Table V. *Electrophoretic mobilities of fractions A and B*

| $p$H | Mobility cm./sec. $10^{-5}$ Fraction A | Mobility cm./sec. $10^{-5}$ Fraction B | Direction |
|---|---|---|---|
| 4·19 | 9·35 | 8·47 | Cathodic |
| 4·36 | 5·56 | 5·88 | ,, |
| 4·66 | 1·81 | — | ,, |
| 4·67 | — | 1·68 | ,, |
| 4·97 | 1·51 | 2·25 | Anodic |
| 5·27 | 4·19 | — | ,, |
| 5·28 | — | 4·39 | ,, |
| 5·48 | 5·80 | — | ,, |
| 5·49 | — | 5·95 | ,, |

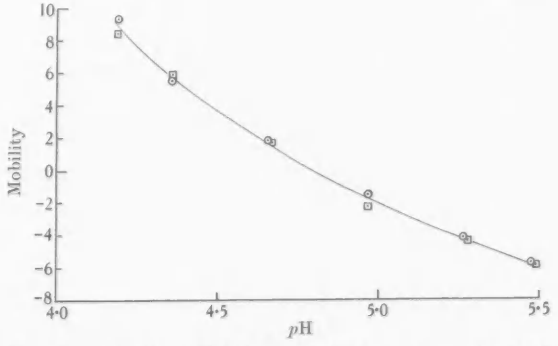


Fig. 2. The electrophoretic mobilities of crystallized horse serum albumin fractions. $T = 20°$. Fraction A: ⊙. Fraction B: ⊡.

An examination of the figure reveals that no significant differences occur in the electrophoretic mobilities of the two fractions over the *p*H range studied. It remains possible, however, that at *p*H farther removed from the isoelectric point differences might be found in the mobilities of the fractions although this is not apparent at *p*H 8·03 [Tiselius, 1937].

The curves obtained during the investigations again showed no sign of heterogeneity in either fraction.

The value obtained for the isoelectric point, *p*H 4·80, is lower than that found by Tiselius [1930] (*p*H 4·88) for horse serum albumin, but agrees excellently with an extensive series of results obtained by Pedersen (unpublished) using the same method, and which also indicate an isoelectric point *p*H 4·80 for horse serum albumin. For a product isolated electrophoretically, Tiselius [1937] reports a value *p*H 4·64 for the isoelectric point of horse serum albumin, using a modified method. This latter value is not directly comparable as it was determined at $T = 0°$ and at an ionic strength $\mu = 0{\cdot}1$, whereas for the value reported in this paper $T = 20{\cdot}0°$ and $\mu = 0{\cdot}02$, as in Pedersen's measurements.

*Diffusion constant measurements*[1] *and molecular weights.*

Determinations of the diffusion constant for each fraction were made by the method of Lamm & Polson [1936]. Two concentrations of protein were used in each case, and the data were analysed by equations (2) and (4 *a*) of Lamm & Polson's paper. The mean values corrected to pure water at 20° are given in Table VI.

Both fractions were shown to be homogeneous, as the normal dispersion curve coincided exactly with experimental curves for each fraction.

No significant differences appear in the diffusion constants for the two fractions. The actual values found are rather lower than those of Lamm & Polson for horse serum albumin, but the preparation used by them was not completely homogeneous. They agree however with the value quoted by Svedberg [1937].

Table VI. *Diffusion constants and molecular weights of fractions A and B*

| Fraction | Protein g./100 ml. | $D$ corr. cm.²/sec. $\times 10^7$ | $S_{20} \times 10^{13}$ Mean | $M$ |
|---|---|---|---|---|
| A | 0·425 | 6·12 | 4·44 | 70,400 |
| A | 0·850 | 6·01 | | |
| B | 0·720 | 6·28 | 4·48 | 70,200 |
| B | 1·44 | 6·01 | | |

$T = 20{\cdot}0°$.

In Table VI are included also the molecular weights ($M$) of the two fractions calculated from the equation:

$$M = \frac{RTs}{(1 - V\rho)} \qquad \text{[Svedberg, 1925].}$$

The mean values for the sedimentation constants and diffusion constants reported for the fractions in this paper were used, and the partial specific volume was taken as 0·748 in each case.

The values obtained for $M$ agree with those quoted by Svedberg [1937].

[1] I am indebted to Mr A. G. Polson for making these determinations.

## Discussion

The two main points arising for discussion would appear to be the relation of the results presented here to the recent findings of Hewitt [1936; 1937], and the significance of the physico-chemical data.

Hewitt has isolated a crystallizable albumin of low carbohydrate content from horse serum and also an amorphous material of high carbohydrate content. These he calls crystalbumin and seroglycoid respectively.

There seems little doubt that the fraction B of this paper and Hewitt's crystalbumin are identical, despite the fact that the nitrogen content of crystalbumin (14·4 %) is considerably lower than that found here (15·16 %). The latter figure is in much better agreement with the determination of Adair & Robinson [1930] on "crystalline serum albumin", viz. 15·6 %. With a molecular weight of 70,200 for this material, a carbohydrate content of 0·083 % leads to a value of 58 g. carbohydrate per mol. protein, or about one-third of a hexose residue per mol. protein. The conclusion seems justified that this material contains no carbohydrate residue, but that the orcinol reacts with some grouping other than carbohydrate in this particular protein, under the conditions of the experiment. Glucosamine gives no colour with the orcinol reagent and its presence is not excluded.

The data obtained during the recrystallization of fraction A suggest strongly that it too is a chemical individual. Its carbohydrate content, 1·95 % about 8 hexose residues per mol. protein, is considerably lower than that of Hewitt's amorphous seroglycoid.

No quantitative significance is claimed for these carbohydrate values in view of the arbitrary nature of the reference solution.

It has already been pointed out that the mother liquors from crystallization 2 of fraction A have a higher carbohydrate:nitrogen ratio than the crystals. Further, the value of this ratio is greater than that of serum globulin, of which a small amount was shown to be present by ultracentrifugal analysis. Consequently if one accepts the chemical individuality of fractions A and B, at least one more non-dialysable material of high carbohydrate content must be assumed present to account for the facts.

It has been shown [Pedersen & Waldenström, 1937] that bilirubin migrates with serum albumin both in the ultracentrifuge and electrophoretically. In the experiments described here only fraction A was associated with a yellow pigment, probably bilirubin, fraction B being colourless.

It is of considerable interest that no significant differences were observed in the sedimentation constants, diffusion constants and electrophoretic mobilities of the two fractions, which are manifestly chemically differentiated. It would appear that the problem here is beyond the present analytical capabilities of these physico-chemical methods.

A further differentiation between easily soluble and less soluble fractions of crystalline serum albumin is shown by the dielectric constant increment [Oncley & Ferry, quoted by Cohn, 1937], values of 30,000 and 11,000 having been found for such fractions respectively.

Some preliminary measurements on the solubility of fraction B in aqueous sodium sulphate indicate that the quantity of protein dissolved is dependent on the amount of solid phase present. This agrees with the findings of Sørensen [1930], but is surprising in view of the variety of criteria of purity to which the substance has been shown to conform in this paper.

SUMMARY.

1. The crystallization and fractionation of horse serum albumin is described, using sodium sulphate as a precipitating agent.

2. Evidence regarding the chemical individualities of two crystalline fractions of horse serum albumin is presented.

3. A survey of some physico-chemical characteristics of the fractions is given and certain limitations of the methods pointed out.

The author wishes to acknowledge the generous hospitality of Prof. Svedberg. In Sweden parts of the costs of this investigation were defrayed by grants from the Andersson Foundation, the Nobel Foundation and the Rockefeller Foundation. In England the costs were defrayed by a grant from the Government Grants Committee of the Royal Society.

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## APPENDIX

### ON THE SEROLOGICAL PROPERTIES OF THE ALBUMIN FRACTIONS

BY P. G. H. GELL AND M. E. YUILL

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The purpose of the following experiments was to distinguish serologically between the two albumin fractions described above.

Antisera to fraction A (carbohydrate-rich), fraction B (carbohydrate-poor) and, as a control, to a specimen of thrice recrystallized horse serum albumin (from a different animal), were prepared as follows. Three rabbits were used for

each antigen; for 3 weeks the animals received tri-weekly intravenous doses of antigen, starting with a 10 mg. dose and increasing by 10 mg. each week, thus receiving 180 mg. in all. Serum was collected on the 8th day after the last injection, and stored at 2° without preservative.

*Ring tests.* Tenfold dilutions of antigen were layered on the surface of neat antiserum, and the appearance of a disk of precipitate at the serum-antigen interface noted after about 30 min. at 20°. With all three antisera, the reactions of the heterologous antigens were nearly as marked, as regards degree and end-point, as those of the homologous antigens. In addition, two zones of maximum precipitation occurred in some heterologous and homologous systems. The production of strong antisera to fraction B (carbohydrate-poor) does not confirm the findings of Hewitt [1937].

*Bulk tests.* The crude ring test results were checked by bulk tests, in which equal volumes of antiserum (1/5) were incubated at 37° with each of a threefold dilution-series of antigen.

Table I shows the optimal flocculating concentration of the antigen: the optima recorded appeared at widely different times, sometimes after 15 min. and sometimes only after 24 hr. incubation.

Table I. *Bulk precipitin tests. Optimal reacting concentrations of three antigens with the three antisera*

Antigens: Recrystallized albumin ... R. Anti-R ... Antisera
Carbohydrate-rich fraction ... A. Anti-A
Carbohydrate-poor fraction... B. Anti-B

| | Optimal flocculating concentrations of antigen | | | | | |
|---|---|---|---|---|---|---|
| Antisera 1/5 | Antigen R | | Antigen A | | Antigen B | |
| Anti-R | 1/320, | 1/38400 | 1/5120 * | | 1/80, | **1/40960** |
| Anti-A | 1/200, | 1/25600 | **1/540** | | 1/20, | 1/14580 |
| Anti-B | 1/100, | 1/6400 | 1/3840 * | | 1/20, | **1/4860** |

* Indicates wide zone of flocculation.

Thick type indicates optima with rapid and copious precipitation.

It will be seen that several of the systems show two clearly marked optimal reacting concentrations of antigen; this may be taken as evidence that, in these at least, more than one antigen-antibody reaction is taking place [cp. Dean *et al.* 1935; Taylor & Adair, 1935; Goldsworthy & Rudd, 1935].

*Precipitin absorption tests* were then attempted, by absorbing the three antisera with heterologous antigen in a concentration corresponding to the optimum, or, in cases in which two zones appeared, to the optimum occurring with the more concentrated antigen. The mixtures were held at 37° for 20 hr., the precipitates centrifuged down, and the supernatants tested against antigen solutions as in Table II.

It will be seen that as far as these absorption tests go, R and A behave as though they were identical, and B appears to be lacking in an antigenic component possessed by R and A. This result suggests that the carbohydrate partly determines the antigenic specificity of horse serum-albumin (R) and of the

Table II. *Precipitin absorption tests. Supernatants of optimal reactions tested in ring test for specific antibodies*

| Antiserum | Absorbed by antigen | Supernatant tested with antigen | | |
|---|---|---|---|---|
| | | R | A | B |
| Anti-R | ... A | – | – | – |
| Anti-R | ... B | + | + | – |
| Anti-A | ... R | – | – | – |
| Anti-A | ... B | + | + | – |
| Anti-B | ... R | – | – | – |
| Anti-B | ... A | – | – | – |

carbohydrate-rich fraction (A), and though the capacity to stimulate anti-carbohydrate antibodies has not necessarily been wholly lost by the carbohydrate-poor fraction (B), yet it has little power to absorb such antibodies from anti-R and anti-A sera.

Such an interpretation must be accepted with caution, for it fails to account fully for the distribution of double-zones recorded in Table I. The evidence of double zones, and of other tests now in progress, suggests an antigenic pattern of greater complexity.

We desire to thank Prof. A. A. Miles for his help in analysing the results of the serological tests.

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# LXXIII. STUDIES ON BACTERIAL PHOSPHATASES

## III. THE PHOSPHATASES OF *AEROBACTER AEROGENES, ALCALIGENES FAECALIS* AND *BACILLUS SUBTILIS*

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Phosphatases from different tissues show marked differences in their *p*H-activity relationships, and smaller differences in other properties. In some respects such as the effects of various ions, especially magnesium, all phosphatase preparations act similarly, though to a varying extent. While it would be convenient to believe that the mode of action of all orthophosphatases is the same, yet such a hypothesis must await a large accumulation of data. The interest attaching to specificity prompted an extension of the work on bacterial phosphatases already reported [Pett & Wynne, 1933].

This paper indicates the results obtained with three other bacterial species, *Aerobacter aerogenes, Alcaligenes faecalis* and *Bacillus subtilis*, in none of which has this enzyme been reported. These species have a special interest. The previous paper reported phosphatases in a facultative anaerobic, spore-forming, acid- and gas-producing rod (*Clostridium acetobutylicum*), and a non-spore-forming organism (*Propionibacterium jensenii*). This paper uses *Aerobacter aerogenes* as an acid- and gas-producing, spore-forming rod of aerobic type, in contrast to *Clostridium*. *Alcaligenes faecalis* was included because it does not produce acid or gas, and it is doubtful if it attacks carbohydrates. The *B. subtilis* was used because two distinctly different strains were available and it is valuable to see how the phosphatases differ. The *B. subtilis* A is the Marburg strain, and *B. subtilis* B the Michigan strain [Soule, 1932], which have been discussed by the American Society of Bacteriologists in the definition of a type-species of aerobic spore-formers (Genus *Bacillus*).

### *Materials and methods*

Conditions were the same as those described by Pett & Wynne [1933] with the following variations. The reaction volume was only 5 ml. instead of 10, and the dried organisms were used at the rate of 0·03 g. per 5 ml. instead of 0·1 g. per 10 ml. The results have been shown to be comparable, however. In the acid range 0·025 *M* phthalate buffers were used; in the alkaline range either borate or

glycine buffers were used, no difference being observed in the amount of hydrolysis at a given *p*H. In the previous work 1 hr. hydrolysis, which is still on the linear part of the curve, was taken for comparison, but in the present work the activity was such that the curve is falling off slightly, so comparison is made on $\frac{1}{2}$ hr. readings. No toluene of chloroform was used for this short period of digestion.

*Aerobacter aerogenes* was grown at 37° in 3% glucose and 1% peptone for 2 days. Longer times yielded no more organisms.

*Alcaligenes faecalis* was grown at 37° for 4 days in 3% peptone. No increase in yield was obtained by using glucose, or by longer periods.

*B. subtilis* A (the Marburg strain) was grown at 37° for 4–5 days in 4% glucose and 1% peptone. It formed a thick wrinkly pellicle, and the liquid is not turbid if this is left undisturbed. A better yield is obtained if this is shaken down daily.

*B. subtilis* B (the Michigan strain) was grown at 37° for 6–7 days in a medium containing 1% beef extract and 4% glucose. The use of beef extract greatly increased the yield over bactopeptone.

## *Some properties of the enzymes*

(1) p*H-activity.*

Table I shows the *p*H optima of the phosphatases studied in this paper, together with those for most other reported phosphatases, to facilitate comparison. These *p*H optima have been first reported or confirmed by the authors [*v.* Pett & Wynne, 1934].

Table I. *The optimum* p*H for phosphatases from different sources acting on three substrates*

| Source of enzyme | Optimum *p*H on substrate indicated | | |
|---|---|---|---|
| | Glycerophosphate | Hexosephosphate | Pyrophosphate |
| *Aerobacter aerogenes* | 5·6 | 5·6 | 6·2 |
| *B. subtilis* A | 10·7 | 6·6 | 6·6 |
| *B. subtilis* B | 6·5 | 6·0 | 6·2 |
| *Alcaligenes faecalis* | 8·7 | 8·5 | 7·6 |
| *Cl. acetobutylicum* | 5·1 | 6·0 | 7·0 |
| *Prop. jensenii* | 7·0 | 6·0 | 7·0 |
| *Sacc. cerevisiae* | 4·0 | 6·5 | — |
| Takadiastase | 4·0 | 4·2 | — |
| Mammalian tissues | 8·9 | 8·9 | — |
| Erythrocytes | 6·0 | 6·6 | — |
| Soya bean | 5·3 | — | — |
| White bean | 5·8 | — | — |

It must be remembered for any enzyme that small differences in the optimum *p*H are observed from one preparation to another. These are usually of the order of 0·2 *p*H. Furthermore the relation of activity to *p*H is best expressed by a curve with a more or less broad peak. Curves have been established for every enzyme reported in Table I, and the actual peaks must be considered as spread around the figures given by about $\pm 0{\cdot}2$ *p*H, followed by a rapid falling away on each side.

(2) *Effects of various ions.*

Previous work of the authors showed that phosphatases of two species of bacteria, in common with mammalian phosphatases, are stimulated by magnesium, inhibited by free phosphate, and respond to other ions in various ways. Similar studies have been carried out in the present case. Several factors must be

considered in such experiments where substances are added to enzyme digests—and much work on enzymes has not recognized these factors. It must be emphasized that the addition of any substance to an enzyme digest is liable to alter the *p*H, even in the presence of buffers. In the present work all solutions were adjusted to the desired *p*H before mixing and the *p*H after hydrolysis was determined. A variation of 0·3 unit discredited the experiment. The phosphate estimations were made with Fiske & Subbarow's method [1925] which is less sensitive to salts in solution than methods employing different reducers. In the present experiment it must also be admitted that in the alkaline range magnesium tends to precipitate and its exact concentration is doubtful.

The results with various ions may be summarized as follows. Magnesium, at some concentration, accelerated the rate of hydrolysis in every instance except with *B. subtilis* B. A very broad curve is the usual sign of this effect. As with the organisms previously studied, zinc, in low concentration, stimulated activity on pyrophosphate, except with *aerogenes*. Fluoride, in some concentration, was strongly inhibitory to glycerophosphatase, fairly inhibitory to pryophosphatase, and slightly so to hexosephosphatase, except with *aerogenes*. In this respect *aerogenes* resembles the organisms previously studied. Cyanide and calcium were without effect except in high concentrations ($M/10$). Phosphate was, as theory demands, inhibitory.

## Discussion

This work is of interest in three ways: (1) the specificity of phosphatases, (2) the presence of a phosphatase in an organism (*Alcaligenes faecalis*) containing no glycolytic mechanism and (3) the marked differences between the two strains of *B. subtilis*.

Regarding specificity no conclusions can be drawn. Consistent differences in *p*H-optima both among bacterial phosphatases and these compared with other sources of enzyme form a striking argument for some kind of substrate and source specificity. Broadly speaking there is a similarity in the effects of various ions, especially magnesium, among all bacterial phosphatases. The differences observed may be due to the crudity of the preparations, though this is not necessarily the case.

Significant differences were found between the phosphatases of the two strains of *B. subtilis* (Marburg & Michigan). Not only are reactions to magnesium and the *p*H-optima distinctly different, but also the degree of inhibition by fluoride is much greater with the B (Michigan) strain, a fact possibly related to their different fermentation reactions. Undoubtedly biochemical differences of this sort will prove more and more valuable in differentiating bacterial species as they become worked out.

## Summary

1. As an extension of previous work the phosphatase systems of *Aerobacter aerogenes*, *Alcaligenes faecalis*, and two strains of *Bacillus subtilis* (Marburg & Michigan) have been studied. Activity-*p*H curves on various substrates and the effects of magnesium, phosphate and other ions on the rate of hydrolysis were determined.

2. The significance of the observations has been discussed in relation to (1) the specificity of phosphatases, and (2) the difference between the two strains of *B. subtilis*.

3. It is noteworthy that *Alcaligenes faecalis* contains an active phosphatase since it lacks a glycolytic system.

The authors are grateful to Prof. W. L. Holman for cultures of the two strains of *Bacillus subtilis*, to Prof. D. T. Fraser for a culture of *Aerobacter aerogenes*, and to Mr E. C. Beck for a culture of *Alcaligenes faecalis*. The work was carried out at the University of Toronto.

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# LXXIV. THE METABOLISM OF BONE MARROW

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In the course of work on histological changes in the bone marrow of rabbits, it became of interest for various reasons to know to what metabolic type this tissue belongs. Search of the literature revealed that very little is known on this subject. The metabolism of leucocytes from blood (both normal and leukaemic) has been considerably studied [Fleischmann & Kubowitz, 1927; Barron & Harrop, 1929; Glover *et al.*, 1930; Soffer & Wintrobe, 1932], but the cells in marrow from which some of these are derived are not necessarily identical in their behaviour. The presence of the nucleated precursors of the erythrocytes may also be expected to affect the metabolism of marrow.

With regard to whole bone marrow the only measurements we have found are those of v. Bréza [1926], who, using teased preparations, found that in the rabbit $Q_{O_2} = 3{\cdot}5$–$5{\cdot}0$ and in the guinea-pig $Q_{O_2} = 4{\cdot}0$–$6{\cdot}0$, reckoned on the fat-free dry wt.

In addition Fujita [1928] used cells from the bone marrow of the rat, selecting those cells which escaped into the medium when the marrow was gently stirred with serum or Ringer. In serum he found that such cells have a metabolism roughly of the type characteristic of embryonic tissues ($Q_{O_2} = -9{\cdot}2$, $Q_{CO_2}^{O_2} = +3{\cdot}7$, $Q_{CO_2}^{N_2} = +20{\cdot}1$), but their behaviour in Ringer was quite different. Here the same relationship was true initially ($Q_{O_2} = -12{\cdot}9$, $Q_{CO_2}^{O_2} = +3{\cdot}8$, $Q_{CO_2}^{N_2} = +25{\cdot}8$), but in the course of 3 hr. the respiration decreased while the aerobic glycolysis increased (final values $Q_{O_2} = 0{\cdot}0$, $Q_{CO_2}^{O_2} = +17{\cdot}5$, $Q_{CO_2}^{N_2} = +18{\cdot}9$). This was interpreted by Warburg [1929] as an inhibition of the respiration by the Ringer's solution.

## Experimental

*Preparation of material.* The whole of the marrow from six bones (femora, tibiae and humeri) of a rabbit (except for a small portion taken for histological examination) was removed as rapidly as possible, placed in about 20 ml. of Ringer in a 50 ml. flask, and shaken gently for a few minutes. The marrow rapidly broke up into pieces small enough to give fairly accurate sampling and to give reliable measurements of metabolism, according to the criteria of Warburg [1923]. v. Bréza [1926] states that suspensions of marrow should not be used for measurements of respiration, on account of the instability of the cells. We have seen no sign of metabolic instability in our experiments, and in most cases found the metabolic reactions to proceed linearly (see below).

*Calculation of results.* The usual method of expressing these results, viz. in terms of dry wt. of tissue, cannot be satisfactorily applied to marrow. This tissue, in the rabbit, consists predominatingly of adipose tissue in which the fat is metabolically inactive, and moreover the proportion of fat in different normal marrows varies very widely. To overcome this difficulty, it was decided to estimate the amount of cellular material in the marrow samples by determination of

the total N content. To establish the relationship between fat-free dry wt. and N content, marrow samples from 10 rabbits were analysed, with results given in Table I.

Table I

| Sample | Fat % of dry wt. | Nitrogen % of fat-free dry wt. |
|---|---|---|
| 1 | 71 | 14·6 |
| 2 | 70 | 13·9 |
| 3 | 71 | 14·5 |
| 4 | 88 | 15·2 |
| 5 | 71 | 15·0 |
| 6 | 90 | 14·6 |
| 7 | 89 | 14·2 |
| 8 | 84 | 14·6 |
| 9 | 91 | 15·2 |
| 10 | 88 | 13·9 |
| | Average | 14·6 |

While the wt. of the non-fatty residue varied between 9 and 30 % of the dry wt. of the sample, the N content of this residue varied only between 13·9 and 15·2 %, with an average of 14·6 %.

At the end of every experiment the contents of the manometer vessels were washed out into Kjeldahl flasks and the total N determined in the usual way. The figure for fat-free dry wt. of tissue was obtained by multiplying the N by 6·85. In experiments where the lactic acid formed was estimated chemically, the solution was deproteinized and the N determination made on the protein precipitate. Trichloroacetic acid was found to be unsuitable as precipitant, the chemical estimations in such filtrates being invariably too low. This was attributed to solution of a part of the lactic acid in the fat, for when a neutral precipitant was used, so that the lactic acid remained dissociated, satisfactory results were obtained. The procedure used was to wash out the contents of the Barcroft vessels with a mixture of 15 ml. 0·75 % $ZnSO_4$, $7H_2O$ and 5 ml. 0·1 *N* NaOH and heat the solution in a boiling water bath for 5 min. In these filtrates the lactic acid was determined by the method of Friedemann *et al.* [1927], and the results showed satisfactory agreement with the manometric figures.

*Measurement of respiration, aerobic glycolysis and anaerobic glycolysis.* The procedure was exactly that described by Berenblum *et al.* [1936], respiration being measured in phosphate Ringer and glycolysis in bicarbonate Ringer, all in a total volume of 5 ml. in Barcroft manometers. The marrow suspension was measured from a special wide-tipped 2 ml. pipette.

*Histological methods.* Small portions of marrow were fixed in 4 % formaldehyde saline, embedded in paraffin, and sections were stained by Harris's haematoxylin and aqueous eosin, and by Turnbull's modification for paraffin sections of Jenner's stain. The relative proportions of the various cells were estimated independently in the sections stained by each method, and in most cases the results were found to agree closely. Previous work has shown that it is unusual for the relative numbers of the cell types to vary significantly between different parts of the marrow of the same animal, so we feel justified in basing the histological classification of these marrows on a small sample, reserving most of the marrow for the metabolism experiments.

*"Normal" and "abnormal" marrow.* Like most tissues bone marrow contains a large number of different kinds of cells, which may vary greatly in their metabolism, but unlike many other tissues the proportion of these different cells may vary considerably in different normal animals, and may be made to vary

even more by various treatments. We have used marrow from animals treated in the following ways.

(1) Intravenous injection of nucleic acid or the nucleotides derived therefrom by alkaline hydrolysis. This causes a blood leucocytosis, the marrow being depleted of granulocytes. During the recovery phase haemocytoblasts are increased.

(2) Injection of saponin, which appears to have a direct toxic effect on the marrow, both granulocytes and normoblasts being liberated into the circulation.

(3) Injection of benzene, which progressively destroys the marrow cells. The leucopoietic tissue is more vulnerable than the erythropoietic. The main effect appears to be on the myelocytes and their precursors.

(4) Exposure to an atmosphere of CO calculated to produce half saturation of the haemoglobin, which leads to hyperplasia of the erythropoietic cells.

(5) Grafting of the Brown-Pearce carcinoma. When dissemination of the growth has taken place, the marrow is found to be hyperplastic [Orr, 1936]. The hyperplasia is more often leucopoietic than erythropoietic.

The normal distribution of the various cell types in rabbit bone marrow is fairly well defined, the ratio of erythropoietic to granulopoietic tissue averaging $\frac{1}{2}$ to $\frac{1}{3}$, and animals in which this ratio was outside these limits have been judged "abnormal" for the purposes of this investigation. Usually the differentiated cells constituted all but a very small fraction of the haemopoietic tissue, and when haemocytoblasts were numerous the marrow was "abnormal".

## Results

*Normal marrow.* Results for "normal" marrow were obtained not necessarily from normal (that is, untreated) rabbits, but from any rabbits which, whether treated or untreated, showed a "normal" histological picture in their bone marrow. Of the 29 rabbits studied, 8 gave marrow which satisfied the criteria of "normality", and the metabolism of these is given in Table II.

Table II

| Rabbit | Treatment | $Q_{O_2}$ | $Q_{CO_2}^{O_2}$ | $Q_{CO_2}^{N_2}$ |
|---|---|---|---|---|
| 26. ii. 35 | — | −4·5 | +4·8 | +9·0 |
| 582 | Nucleic acid + benzene | −3·7 | +1·9 | +4·2 |
| 580 | Nucleic acid + benzene | −4·1 | +2·8 | +6·7 |
| 27. ii. 35 | — | −5·0 | +3·9 | +6·6 |
| 542 | Saponin | −3·7 | +4·8 | +8·2 |
| 12. iv. 35 | — | −3·4 | +3·5 | +8·0 |
| 547 | — | −2·9 | +3·4 | +6·3 |
| 586 | Brown-Pearce carcinoma | −3·5 | +5·0 | +8·2 |
| | Average | −3·9 | +3·8 | +7·1 |

It will be seen that the values of $Q_{O_2}$ with one exception fall within the range of values given by v. Bréza, and that the ratios $Q_{O_2} : Q_{CO_2}^{O_2} : Q_{CO_2}^{N_2}$ are of the order 1:1:2. The phenomenon reported by Fujita [1928] has never been observed. The metabolic rates were commonly linear for the 1st hr. and respiration, aerobic glycolysis and anaerobic glycolysis gradually decreased coincidently if the experiment was continued longer (see Fig. 1, where results up to 4 hr. are given). On occasion the falling off in velocity started earlier, but though in some cases the respiration seemed to be more unstable than the glycolysis an associated increase in the aerobic glycolysis was never found.

In one experiment marrow from two rats was used, and again all three metabolic rates were constant for the 1st hr., and showed approximately equal rates of decrease during 3 hr. (initial $Q_{O_2} = -5{\cdot}8$, $Q^{O_2}_{CO_2} = +8{\cdot}6$, $Q^{N_2}_{CO_2} = +10{\cdot}8$).

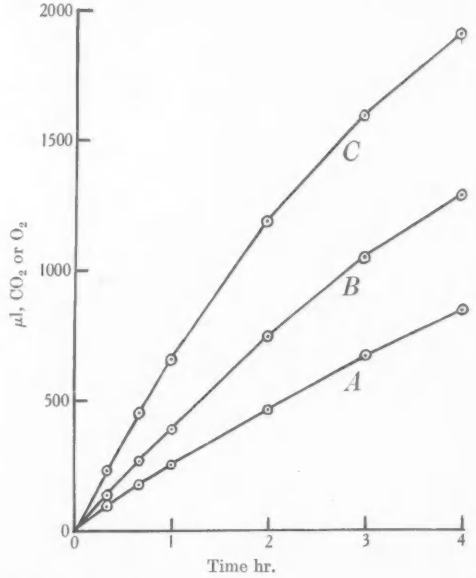


Fig. 1. The metabolism of bone marrow: *A*, respiration, *B*, aerobic glycolysis, *C*, anaerobic glycolysis.

*"Abnormal" marrows.* Our object was to decide what part of the total metabolism of marrow was contributed by the various types of cells present in it, and the method used was to vary the proportions of these types by applying to the rabbits the stimuli described. It was found, however, that the amount of

Table III. *Metabolism of "abnormal" marrows*

| Type of marrow | No. of rabbits | Average $Q_{O_2}$ | Average $Q^{O_2}_{CO_2}$ | Average $Q^{N_2}_{CO_2}$ |
|---|---|---|---|---|
| Rich in granulocytes* | 5 | −4·5 | +5·7 | +9·5 |
| "Normal" in granulocytes | 8 | −3·9 | +3·8 | +7·1 |
| Deficient in granulocytes | 16 | −3·5 | +1·9 | +4·5 |
| Rich in nucleated red cells† | 12 | −3·7 | +2·2 | +5·1 |
| "Normal" in nucleated red cells† | 9 | −3·8 | +3·4 | +6·4 |
| Deficient in nucleated red cells | 8 | −3·8 | +4·1 | +7·4 |
| Rich in other cells‡ | 14 | −3·5 | +2·0 | +4·5 |
| "Normal" in other cells | 14 | −3·9 | +3·8 | +7·3 |
| Deficient in other cells | 1 | −4·7 | +6·7 | +11·4 |

* Myelocytes and polymorphonuclear leucocytes in varying proportions.
† Erythroblasts and normoblasts in varying proportions.
‡ Mostly haemocytoblasts (primitive cells not differentiated into either erythroblasts or granulocytes).

variation that could be achieved was insufficient to provide really conclusive results, without using very large numbers of experiments so that statistical methods could be applied. The results from 29 rabbits do nevertheless show some rough indications, and we give them for what they are worth.

In each case the condition of the marrow was judged by histological examination of a small sample, and the classification was carried out by one of us (J. W. O.) without knowledge of the results of the metabolic experiments.

## Discussion

Our results show that the bone marrow of rabbits falls into the same class, as regards its metabolism, as tumour tissue, retina and kidney medulla, i.e. the ratios $Q_{O_2} : Q^{O_2}_{CO_2} : Q^{N_2}_{CO_2}$ are roughly 1 : 1 : 2. The absolute value of the metabolism is small, which is rather surprising seeing that it is an actively growing tissue. During phases of active growth (e.g. in rabbits bearing the Brown-Pearce carcinoma) the metabolism of the whole marrow is raised in virtue of the increased cell content, but the results on the basis of N content are only slightly higher than normal.

The effect on this metabolism of changes in the distribution of the different types of cells in the marrow is not very clear, but the figures justify the generalization that, while all the cells contribute to the respiration of the tissue, the greater part of the glycolysis is to be attributed to the granulocytes. In one experiment, where the glycolysis was nil, histological examination of the marrow showed it to be practically free from granulocytes; the conditions could not be repeated, however, so it would be unwise to attach undue importance to this observation.

We can explain the results of Fujita [1928] only by supposing that the cells washed out from bone marrow by stirring with serum or Ringer's solution are only a small part of the total cells of the tissue, and in their metabolic instability are far from typical of the tissue as a whole.

## Summary

The metabolism of the bone marrow of rabbits is found to be of the same type as that of tumour tissue, retina and kidney medulla. The average values for normal marrow are:

$$Q_{O_2} = -3{\cdot}9,\ Q^{O_2}_{CO_2} = +3{\cdot}8 \text{ and } Q^{N_2}_{CO_2} = +7{\cdot}1.$$

Indirect measurements indicate that probably most of the glycolysis is due to the granulocytes, while these cells and the nucleated red cells are about equally active in their respiration.

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# LXXV. STUDIES IN GLYCOLYSIS
## II. THE FIRST STAGES OF GLYCOLYSIS IN MUSCLE EXTRACTS

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In a previous communication [Kendal & Stickland, 1937, 1] it was shown that if rabbit muscle is minced and thoroughly washed with water, and then extracted several times with dilute $Na_2HPO_4$, the later extracts (usually the 4th) contain much of the glycolytic enzyme system but are freer from coenzymes than extracts prepared by earlier methods. This was shown by the fact that on addition of boiled muscle extract a rapid formation of lactic acid from polysaccharide took place, while on addition of $Mg^{++}$, adenosinetriphosphate, cozymase and hexosediphosphate practically no lactic acid was formed. It was also shown that if $Mg^{++}$ and adenosinetriphosphate alone were added to such extracts considerable quantities of phosphate were esterified, although no lactic acid was formed. The present communication deals with the nature of the esters formed under these conditions.

The initial esterification process in the glycolytic enzyme system prepared by the method of Meyerhof [1925] has been studied by Parnas and his collaborators [Parnas & Baranowski, 1935; Ostern *et al.* 1936]. They found that a direct reaction between glycogen and inorganic phosphate takes place, giving rise to the Embden ester. Later Parnas & Mochnacka [1936] showed that by suitable treatment (electrodialysis at *p*H 8·2, or autolysis at 20° followed by long dialysis) extracts could be obtained which no longer phosphorylysed glycogen except on addition of adenylic acid or inosinic acid. Since the original extracts were thought to be free from adenosine phosphates it was supposed that they must have contained inosinic acid. As will be seen later esterification may be brought about by a concentration of adenosinetriphosphate or adenylic acid much too low to be detectable by chemical methods.

Cori & Cori [1936; 1937] discovered, as an intermediate step in the formation of the Embden ester, a new ester which they identified as an aldose-1-monophosphate, and later as phosphoric acid α-glucoside. This ester was formed under the action of washed muscle or pulp or ordinary dialysed (17 hr.) aqueous muscle extract, and was rapidly transformed by the same enzyme preparations into the ordinary Embden ester. The addition of adenylic acid increased the rate of formation of this new ester.

Our experiments with the new enzyme preparations confirm and extend the results of Parnas and of Cori & Cori.

### Experimental

*Preparation of extracts.* The enzyme solutions were made as described in the previous paper [Kendal & Stickland, 1937, 1]. The 4th phosphate GP4 extract was used in nearly all the experiments, but for some purposes the 5th (GP5) and in one the 6th (GP6) proved more suitable.

*Method of experiment.* The solution incubated contained 2 ml. of the enzyme solution in a total volume of 5 ml. It also contained 0·5 % starch or glycogen, or other substrate, 0·25 % $NaHCO_3$, about 3·5 mg. inorganic P per 5 ml. and the necessary coenzyme solutions. All additions were brought to *p*H 7·5. The mixtures were incubated in an atmosphere of $N_2$+5 % $CO_2$ at 37° unless otherwise stated, Barcroft manometer vessels being used for convenience. At the end of the experiment the contents of each vessel were made up to 25 ml. with 5 % trichloroacetic acid and filtered.

In some cases, when the time course of the reaction was to be followed, a large portion of the reaction mixture (usually 30 ml.) was made up in a 100 ml. flask. Four such flasks could be incubated with shaking and continuous gassing on a special stand in the water bath: 5 ml. samples were withdrawn at the required times and made up to 25 ml. with 5 % trichloroacetic acid. As a check against possible evaporation from the solution, in several experiments total P estimations were done on all the samples, and it was found that no appreciable change occurred.

*Determinations.* All P determinations were done by the method of Fiske & Subbarow [1925]. Initial values were obtained either by determinations on the separate components of the mixtures, or by making up a blank experiment and treating it at once with trichloroacetic acid.

The total amount of ester formed was measured by the decrease in organic P, glucose-1-monophosphate[1] by the P liberated on hydrolysis for 10 min. in *N* HCl at 100°, and hexose-6-monophosphates by the difference between these two. This assumption that no ester but glucose-1-monophosphate and Embden ester was formed, was justified in two ways. First, hexosediphosphate was shown not to be formed by testing for alkali-labile P (triose-P), which was invariably absent. This test depends for its validity on the presence of sufficient cozymohexase to allow the equilibrium to be rapidly reached. That the extracts satisfy this condition was shown in our previous paper [Kendal & Stickland, 1937, 1] and it has since been confirmed. Secondly, the ester not hydrolysed in 10 min. was hydrolysed in 4 hr. at 100° by *N* HCl to the extent of 30 % (for the Embden ester Cori & Cori give 28 %, Ostern *et al.* 34·5 % and our own results 25 %). When a large amount of Embden ester was present, the results were corrected to allow for the fact that 3 % of this ester-P is hydrolysed in 10 min. in *N* HCl at 100°.

In experiments with adenosinetriphosphate the results are worked out on the assumption that no ATP is hydrolysed in the course of the experiment. This is not a valid assumption, but the error introduced is not appreciable except in experiments with very high initial ATP concentration and long incubation time.

## Results

### *The formation of CE from carbohydrate*

*The indispensability of coenzymes.* The enzyme-substrate-buffer mixture was incubated with $MgCl_2$, with ATP and with the two together. The concentrations and the results are given in Table I.

[1] The following abbreviations will be used:

| | | |
|---|---|---|
| Glucose-1-monophosphate | = Cori ester | = CE |
| Hexose-6-monophosphates | = Embden ester | = EE |
| Hexosediphosphate | | = HDP |
| Adenosine triphosphate | | = ATP |
| Adenylic acid | | = AA |
| Inosinic acid | | = IA |

Table I

| Enzyme | Coenzymes | Ester formed in 1 hr. (mg. P/5 ml.) Total | CE |
|---|---|---|---|
| GP5 | $MgCl_2$, $M/250$ | 0·01 | 0·00 |
| | ATP, $M/10{,}000$ | 0·09 | 0·09 |
| | $Mg^{++}$ + ATP | 0·54 | 0·54 |
| GP4 | $MgCl_2$, $M/250$ | 0·00 | 0·00 |
| | ATP, $M/20{,}000$ | 0·10 | 0·07 |
| | $Mg^{++}$ + ATP | 0·51 | 0·51 |

GP4 preparations appeared to contain variable amounts of $Mg^{++}$, sometimes enough for considerable activation by addition of ATP alone. On the average, addition of the optimal concentration of $MgCl_2$ caused a threefold increase in the rate of esterification.

Adenylic acid[1] at optimal concentration was found to be as active as adenosinetriphosphate.[2] Inosinic acid showed some slight coenzyme activity at high concentration (Table II).

Table II

| Exp. | Coenzymes | Time of incubation min. | Esters formed (mg. P/5 ml.) Total | CE |
|---|---|---|---|---|
| 1 | $MgCl_2$, $M/250$ | 40 | 0·00 | 0·00 |
| | ,, + ATP, $M/10{,}000$ | 40 | 0·76 | 0·72 |
| | ,, + AA, $M/1000$ | 40 | 0·82 | 0·81 |
| | ,, + IA, $M/1000$ | 40 | 0·12 | 0·08 |
| 2 | $MgCl_2$, $M/250$ + ATP, $M/10{,}000$ | 80 | 0·80 | 0·75 |
| | ,, + AA, $M/2000$ | 80 | 0·83 | 0·78 |
| | ,, + IA, $M/1000$ | 80 | 0·07 | 0·00 |
| 3 | $MgCl_2$, $M/250$ + ATP, $M/7500$ | 120 | 0·85 | — |
| | ,, + AA, $M/2000$ | 120 | 0·84 | — |
| | ,, + IA, $M/1000$ | 120 | 0·06 | — |

*The course of the reaction.* When the amount of ester formed at different times was measured, it was found that the reaction proceeds very rapidly at first, but is approximately linear only for about 10 min., and after 1 hr. is very slow (see Fig. 1). That the reason for this slowing off is not the destruction of the coenzyme is readily shown in four ways. (1) The curve is of the same shape at all ATP concentrations (see Fig. 2). (2) ATP is dephosphorylated only slowly by the enzyme preparation, so that an adequate concentration for maximal esterification is still present even when the reaction has practically stopped. (3) If more ATP is added after 30 min., no increase in esterification is observed over a control (see Fig. 1). (4) If the mixture is incubated with no ATP for 30 min., and ATP then added, very little ester is formed (see Fig. 1). The only conclusion that can be reached is that the enzyme responsible for the conversion of starch into CE is rapidly destroyed during incubation at 37°.

On the other hand Kendal & Stickland [1937, 1] showed that if the enzyme is incubated with $Mg^{++}$ + ATP, the addition of boiled muscle extract at any

[1] We wish to thank Prof. J. K. Parnas for samples of adenylic acid and very pure barium inosinate, and Dr D. M. Needham for another sample of adenylic acid.

[2] In a preliminary communication [Kendal & Stickland, 1937, 2] it was stated that adenylic acid could not replace ATP in this reaction. In arriving at this conclusion we were misled by statements in the literature to the effect that the products of acid hydrolysis of ATP are adenylic acid and orthophosphate. As no pure adenylic acid was available, such hydrolysates (7 min. in $N$ HCl at 100°) of ATP were used. When we became aware of the contrary experience of Parnas & Mochnacka [1936] and Cori & Cori [1936] with adenylic acid, a reinvestigation showed us that the solutions we had previously used contained only adenine and pentosephosphate.

time up to 1 hr. caused rapid lactic acid formation. This reaction must be due initially to lactic acid formation from the preformed monophosphoric esters, though it may be supplemented later by further esterification coupled with the

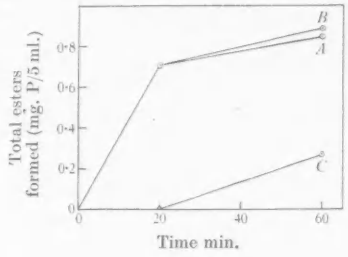


Fig. 1.

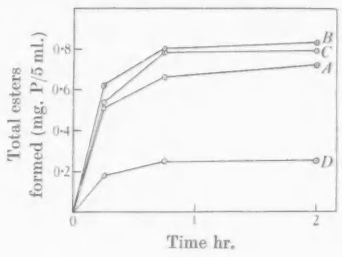


Fig. 2.

Fig. 1. Course of esterification. Curve *A*, ATP ($10^{-4}$ *M*) present from the beginning. Curve *B*, ATP ($10^{-4}$ *M*), further ATP ($10^{-4}$ *M*) added after 20 min. Curve *C*, ATP ($10^{-4}$ *M*) first added after 20 min. incubation.

Fig. 2. Course of esterification at different concentrations of ATP. Curve *A*, $5 \times 10^{-4}$ *M*; Curve *B*, $10^{-4}$ *M*; Curve *C*, $2 \times 10^{-5}$ *M*; Curve *D*, $4 \times 10^{-6}$ *M*.

oxidoreduction reaction between triosephosphate and pyruvic acid [Meyerhof & Kiessling, 1935]. In either case it is clear that the whole enzyme system is stable at 37° except that part which brings about the nucleotide-catalysed phosphorylation of carbohydrate.

The importance of these experiments at the present juncture is merely to show that in kinetic experiments, when ATP is used as coenzyme, incubation periods up to 1 hr. may be used, even though the course of the reaction is not linear, i.e. total esterification in 60 min. is proportional to the initial velocity of esterification.

*Concentration of ATP.* The effect of variation of the concentration of ATP on the rate of esterification was tested in a number of preparations. The $Mg^{++}$ concentration was *M*/250 in every case. All the results obtained with 11 out of 15 preparations tested are given in Fig. 3. It will be noted that 50% of the maximum rate is obtained at a concentration of $10^{-5}$ *M*, and a measurable rate at $2 \times 10^{-6}$ *M*. The other 4 preparations gave points lying to the left of this curve. The significance of this occasional variation in the ATP requirement is not known. It is not due to a more rapid decomposition of ATP, for one preparation which gave a discrepant concentration curve was found to hydrolyse ATP even more slowly than some of those which gave the more usual concentration curve.

*Concentration of AA.* Fig. 3 (curve *B*) shows the effect of varying the concentration of AA at constant $Mg^{++}$ concentration (*M*/250). The amount required is fairly constant in all the preparations tested, and is about seven times that of ATP. This might be due to deamination of AA, for ATP is not deaminated till after dephosphorylation. It is impossible to measure the rate of deamination of AA at the concentrations concerned (*M*/10,000 or less), but indirect evidence suggests that this explanation is the correct one. At high concentrations of ATP or AA, and at low concentrations of ATP, the course of the esterification follows a similar curve, falling off at the same rate in each case. At low concentrations of AA, on the other hand, the esterification comes to a sudden stop

after a very short time (Fig. 4), suggesting that, apart from the inactivation of the enzyme, there has been decomposition of the coenzyme to an inactive product, in this case presumably inosinic acid. It is therefore impossible to say whether the different affinity curves of ATP and AA represent a genuine

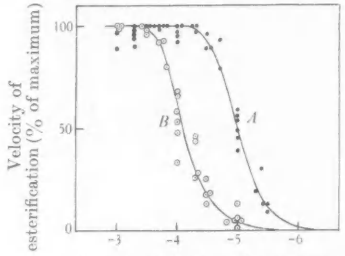


Fig. 3.

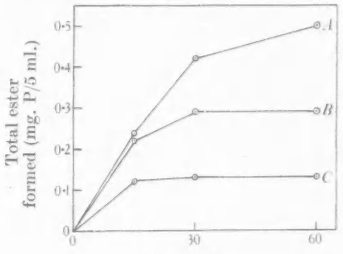


Fig. 4

Fig. 3. Relationship between concentration of coenzyme and velocity of esterification. Curve *A* adenosine triphosphate; Curve *B*, adenylic acid.

Fig. 4. Course of esterification at low concentrations of adenylic acid. Curve *A*, ATP $5 \times 10^{-5}$ *M*; Curve *B*, AA $10^{-4}$ *M*; Curve *C*, AA $5 \times 10^{-5}$ *M*.

difference of affinity, or whether they are entirely accounted for by deamination of AA. Curve *B* of Fig. 3 is certainly to some extent misleading since the velocities recorded at the lowest concentrations of AA are *not* proportional to the initial velocities.

*Concentration of IA.* IA is only very slightly active even at *M*/1000, and at greater dilution of course shows no activity (Table III).

Table III

| Enzyme | Coenzyme | Incubation time min. | Esterification total (mg. P/5 ml.) |
|---|---|---|---|
| GP4 | $Mg^{++}$ + IA, *M*/1000 | 60 | 0·07 |
| | ,, + IA, *M*/3000 | 60 | 0·01 |
| | ,, + IA, *M*/10,000 | 60 | 0·00 |
| | ,, + ATP, *M*/10,000 | 60 | 0·55 |

Mixtures of the three nucleotides show no additive effects (Table IV).

Table IV

| Exp. | Coenzymes* | Incubation time min. | Esterification (mg. P/5 ml.) Total | CE |
|---|---|---|---|---|
| 1 | ATP, *M*/5000 | 20 | 0·55 | 0·54 |
| | AA, *M*/5000 | 20 | 0·49 | 0·45 |
| | ATP + AA | 20 | 0·54 | 0·52 |
| 2 | ATP, *M*/5000 | 60 | 0·74 | 0·66 |
| | IA, *M*/1000 | 60 | 0·06 | 0·02 |
| | ATP + IA | 60 | 0·73 | 0·63 |

* $MgCl_2$, *M*/250 present in all.

*Concentration of Mg.* We have not succeeded in obtaining enzyme preparations which can be presumed to be completely free from $Mg^{++}$ (see Table I).

The effect of increasing additions of $Mg^{++}$ on the rate of esterification is shown in Table V.

Table V

ATP concentration $M/10{,}000$ in both experiments

| Enzyme | Incubation time min. | | Mg concentration (added) ($M$) 0 | $10^{-4}$ | $3\times10^{-4}$ | $10^{-3}$ | $3\times10^{-3}$ | $10^{-2}$ |
|---|---|---|---|---|---|---|---|---|
| GP6 | 30 | Total | 0·17 | 0·22 | 0·29 | 0·43 | 0·52 | 0·62 |
| | | CE | 0·17 | 0·22 | 0·29 | 0·40 | 0·51 | 0·62 |
| GP4 | 60 | Total | 0·04 | 0·10 | 0·13 | 0·18 | 0·30 | 0·39 |
| | | CE | 0·04 | 0·10 | 0·11 | 0·16 | 0·27 | 0·37 |

*Effect of temperature on rate of esterification.* Experiments were carried out in the usual way at 37°, room temperature (20°) and at 0°. The rates of reaction at 37° and at 20° were identical, but at 0° very little esterification occurred (Table VI).

Table VI

| Exp. | Incubation time min. | | 37° | 20° | 0° |
|---|---|---|---|---|---|
| 1 | 60 | Total ester | 0·85 | 0·77 | — |
| | | CE | 0·67 | 0·70 | — |
| 2 | 5 | Total ester | 0·10 | 0·11 | — |
| | 10 | Total ester | 0·21 | 0·20 | — |
| | 20 | Total ester | 0·33 | 0·35 | — |
| | | CE | 0·29 | 0·32 | — |
| 3 | 60 | Total ester | 0·38 | 0·37 | 0·06 |
| | | CE | 0·32 | 0·37 | 0·06 |

*The action of inhibitors of glycolysis.* The three most commonly used inhibitors of glycolysis are iodoacetic acid, fluoride and phloridzin. We have confirmed the findings of Lundsgaard [1933] and Ostern *et al.* [1936] that of these only phloridzin inhibits the initial phosphorylation of carbohydrate, and have further shown that this inhibition operates only on the very first stage, viz. the formation of CE from starch (Table VII).

Table VII

Enzyme GP4; ATP, $M/10{,}000$; $Mg^{++}$, $M/250$. Incubated 60 min.

| Inhibitor | Ester formed (mg. P/5 ml.) Total | CE |
|---|---|---|
| — | 0·75 | 0·55 |
| Sodium fluoride $M/50$ | 0·70 | 0·53 |
| Sodium iodoacetate $M/400$ | 0·72 | 0·55 |
| Phloridzin $M/100$ | 0·07 | 0·00 |

*The identification of CE.* It can be seen in many of the experiments already quoted that with a short incubation period a good yield of almost pure CE is obtained. The quantity of ATP required is so small that the soluble Ba salts should not be contaminated with appreciable amounts of Ba adenylate.

For the isolation of the ester 400 ml. of GP4 were made up to 1 l., containing 0·5 % starch, 0·25 % $NaHCO_3$, $M/250$ $MgCl_2$, $M/10{,}000$ or $M/20{,}000$ ATP and about 700 mg. P as inorganic phosphate mixture at *p*H 7·5. Preliminary experiments showed that variation of the phosphate concentration from this figure in either direction reduced the yield of ester, while variation of the starch concentration between 1·5 and 0·25 % had little effect. The mixture, with $N_2+5$ %

$CO_2$ bubbling through it, was rapidly heated to 37° and maintained at that temperature for usually 30 min. It was then treated with an equal volume of alcohol and enough trichloroacetic acid to make the whole 1 %, cooled thoroughly, allowed to stand for ½ hr. and filtered. The filtrate was neutralized and filtered from a further precipitate of starch, evaporated *in vacuo* to a small volume to remove the alcohol and brought to about 200 ml. with water. Sufficient Ba acetate was added to give a small excess over the total phosphate present, the *p*H adjusted to 9 and the precipitate of Ba phosphate removed by centrifuging. The precipitate was redissolved in HCl and the Ba phosphate reprecipitated at *p*H 9. The two solutions were mixed, treated with alcohol to 60 % and left at 0° overnight, the Ba salt of CE being then centrifuged off and dried *in vacuo*.

This product contained no esters but CE and a trace of EE, but was still contaminated with P-free material of high M.W., its total P content being usually about 6 %. The simplest method for removal of this impurity was found to be by dialysis through collodion. The product, moistened with a little water, was placed in a small collodion bag and dialysed against 100 ml. of water overnight. The Ba salts in the dialysate were then precipitated by the addition of alcohol to 60 %.

The salt could not be dried satisfactorily even over $P_2O_5$ *in vacuo*. It was extremely hygroscopic, and its total P content never rose above 7·4 % (theoretical 7·84 %). When left in a moist atmosphere till constant weight was reached, total P was 6·65 % ($4H_2O$ requires 6·65 %; Cori & Cori [1936] found after drying $3H_2O$). The data for one sample of the Ba salt are given in Table VIII.

Table VIII

| | % |
|---|---|
| Total P (dried over $P_2O_5$) | 7·35 |
| Total P (exposed to water vapour) | 6·65 |
| P hydrolysed in 10 min. in *N* HCl at 100° | 6·75 (92 % of total) |
| Reducing power (as glucose): | |
| Initial | 2·5 |
| Increase on hydrolysis for 10 min.: | |
| Hagedorn and Jensen | 41·0 |
| Hypoiodite [McLeod & Robinson, 1929] | 40·0 |
| Equivalent of P hydrolysed | 39·2 |

Hydrolysis at 70° in *N*/100 HCl:

| Time min. | P % | Sugar % | Cori & Cori (%) |
|---|---|---|---|
| 60 | 42 | 45 | 38 |
| 120 | 65 | 67 | 62 |

Rotation calc. for the anhydrous barium salt, $[\alpha]_D$ ($c = 0{\cdot}3$) = 79°
(Cori & Cori [1936] $[\alpha]_D = 75{\cdot}5°$)

*The formation of hexose-6-monophosphate (EE) from carbohydrate*

In some of the results already given it can be seen that, especially after longer incubation (60 min.), not all the ester formed is hydrolysable in 10 min. If the incubation is further prolonged, an appreciable amount of difficultly hydrolysable ester is found to appear (see Fig. 5), and this ester was readily identified as hexose-6-monophosphate (Embden ester). In our earlier experiments on the action of HDP as a coenzyme in glycolysis it was found that HDP at a concentration of *M*/2000 greatly increases the formation of EE (see Fig. 5). Fig. 5 also shows that the action of HDP is to accelerate the transformation of CE to EE, and not to superimpose the latter on the former. This is more clearly

shown in Fig. 6, where the HDP was added after the formation of CE had been proceeding for 20 min.

The majority of the experiments with hexosediphosphate were carried out with a solution of the rather impure Na salt prepared directly from the commercial Ca salt. A purified sample of the Ba salt, made by taking advantage of its lower solubility in water at higher temperatures, gave a sodium salt which had exactly the same coenzyme properties as that made from the less pure Ca salt.

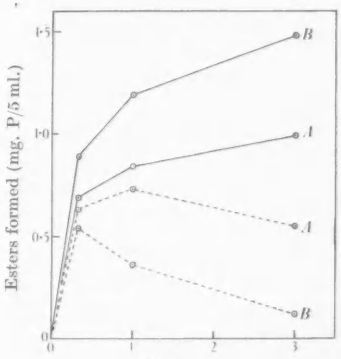


Fig. 5.

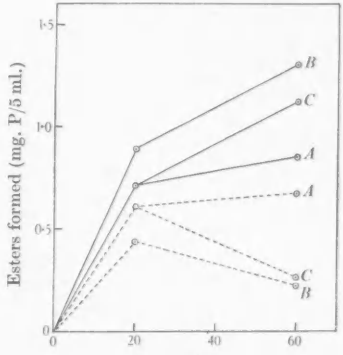


Fig. 6.

Fig. 5. The formation of Embden ester from Cori ester. Curves *A*, HDP absent. Curves *B*, HDP $5 \times 10^{-4}$ *M*. Solid lines, total ester formed; dotted lines, CE formed.

Fig. 6. The conversion of CE into EE. Curves *A*, no HDP. Curves *B*, $5 \times 10^{-4}$ *M* HDP present from the beginning. Curves *C*, $5 \times 10^{-4}$ *M* HDP added after 20 min. Solid lines, total ester formed; dotted lines, CE formed.

The further reaction to EE proceeds of course equally whether the primary esterification was produced by ATP or AA. There is, however, one constant difference between the two cases, viz. the total amount of ester formed with ATP + HDP is considerably greater than with ATP alone, whereas with AA as coenzyme the addition of HDP causes no increase of the total yield of ester (see Table IX). We can offer no explanation of these facts.

Table IX

| Exp. | Coenzymes | (mg. P/5 ml.) Total ester | CE | EE |
|---|---|---|---|---|
| 1 | ATP, *M*/4000 | 0·74 | 0·71 | 0·03 |
| | ,, + HDP, *M*/2000 | 0·98 | 0·49 | 0·49 |
| | AA, *M*/4000 | 0·70 | 0·69 | 0·01 |
| | ,, + HDP, *M*/2000 | 0·73 | 0·23 | 0·50 |
| 2 | ATP, *M*/10,000 | 0·60 | 0·60 | 0·00 |
| | ,, + HDP, *M*/2000 | 0·85 | 0·45 | 0·40 |
| | AA, *M*/10,000 | 0·52 | 0·51 | 0·01 |
| | ,, + HDP, *M*/2000 | 0·42 | 0·10 | 0·32 |
| 3 | ATP, *M*/3000 | 0·62 | 0·57 | 0·05 |
| | ,, + HDP, *M*/2000 | 0·85 | 0·31 | 0·54 |
| | AA, *M*/3000 | 0·58 | 0·43 | 0·15 |
| | ,, + HDP, *M*/2000 | 0·56 | 0·07 | 0·49 |

*The effect of temperature on the rate of the reaction CE → EE*

We have shown that the rate of formation of CE is the same at 20° as at 37°. On the other hand, the rate of conversion of CE into EE is very much greater at 37° than at 20° (Table X). It will be seen that the amount of EE formed from starch at 37° is much greater than that formed at 20°.

Table X

| Exp. | Coenzyme | Temp. °C. | Time of incubation min. | Esters formed Total | CE | EE |
|---|---|---|---|---|---|---|
| 1 | ATP, *M*/20,000 | 37 | 60 | 0·38 | 0·32 | 0·06 |
| | ,, +HDP, *M*/2000 | | | 0·48 | 0·27 | 0·21 |
| | | 20 | 60 | 0·37 | 0·37 | 0·00 |
| | | | | 0·45 | 0·44 | 0·01 |
| 2 | ATP, *M*/10,000 | 37 | 60 | 0·85 | 0·67 | 0·21 |
| | ,, +HDP, *M*/2000 | | | 1·30 | 0·30 | 1·00 |
| | | 20 | 60 | 0·77 | 0·70 | 0·07 |
| | | | | 0·89 | 0·66 | 0·23 |

In the experiments of Parnas & Mochnacka [1936] in which inosinic acid was active as the coenzyme of phosphorolysis, the ester formed was entirely EE. As we find that HDP is necessary for the rapid formation of this ester from CE, there was a possibility that IA+HDP might catalyse the formation of EE in our enzyme preparations, even though IA alone is inactive in the formation of CE. This proved however not to be the case (Table XI).

Table XI

| | Time of incubation (min.) | | | | | |
|---|---|---|---|---|---|---|
| | 40 | | 80 | | 160 | |
| | Esters formed (mg. P/5 ml.) | | | | | |
| Coenzymes* | Total | CE | Total | CE | Total | CE |
| ATP, *M*/10,000 | 0·67 | 0·62 | 0·80 | 0·75 | 0·96 | 0·62 |
| AA, *M*/2000 | 0·72 | 0·72 | 0·83 | 0·78 | 0·93 | 0·65 |
| IA, *M*/1000 | 0·02 | — | 0·07 | — | 0·12 | — |
| IA, *M*/1000+HDP, *M*/2000 | 0·08 | — | 0·09 | — | 0·09 | — |

* $MgCl_2$, *M*/250 present in all.

*The identification of Embden ester*

As was stated earlier, the identity of the difficultly hydrolysable portion of the mixture of esters formed was checked in many experiments by carrying out a 4 hr. hydrolysis in *N* HCl at 100°. The degree of hydrolysis found always agreed with that required for EE (about 30 % in 4 hr.).

To obtain the pure ester, large-scale experiments were set up as described for the preparation of CE, except that HDP was added to a concentration of *M*/2000 and the incubation was prolonged to 2–3 hr. The Ba salt was isolated by the same procedure, except that after the first alcohol precipitation of the ester Ba salts the solution was treated with an equal volume of 2*N* HCl and heated at 100° for 10 min. to hydrolyse the little CE present. The inorganic phosphate liberated by this treatment was removed in the usual way, and the preparation finished as before. The properties of a sample of this salt are given in Table XII.

Table XII

Total P 7·6% (theoretical 7·84%)

Hydrolysis in *N* HCl at 100°:

| Time min. | % hydrolysed | Ostern *et al.* [1936] |
|---|---|---|
| 10 | 3 | — |
| 60 | 11 | 18 |
| 120 | 17 | 25·5 |
| 240 | 25 | 34·5 |

Reducing power:

Alkaline ferricyanide (Hagedorn-Jensen) 32·4% (as glucose)
Hypoiodite [McLeod & Robinson, 1929] 30·1%

Rotation calc. for anhydrous Ba salt ($c = 1·6$), $\alpha_D = 11°$.

The rate of hydrolysis of this ester is, in our experiments, smaller than that reported by previous workers (e.g. Ostern *et al.* [1936]; see Table XII). We have hydrolysed several preparations with constant results (25·3, 25·0 and 25·6% hydrolysis in 4 hr. in *N* HCl at 100°) and think it likely that this lower figure is correct, as earlier workers did not suspect the possibility of contamination with a more easily hydrolysable monophosphate.

*The formation of EE from CE*

Some experiments have been carried out with isolated CE as substrate. A weighed quantity of the Ba salt was dissolved in water, treated with a slight excess of $Na_2SO_4$ and filtered. The amount of conversion was determined by measuring the decrease in the quantity of phosphate hydrolysable in 10 min. in *N* HCl at 100°, and this was checked by carrying out a 4 hr. hydrolysis in *N* HCl at 100° to confirm that the product was EE.

*Indispensability of $Mg^{++}$ and HDP as coenzymes.* $Mg^{++}$ and HDP are both essential for the conversion of CE into EE (Table XIII A), but adenosine phosphates play no part (Table XIII B). A number of other substances, including α-glycerophosphate, phosphoglycerate, EE and glutathione, have been tried in place of HDP, but no other has been found to have any effect on the rate of this reaction. The HDP added in these experiments appears to remain unchanged even after 2 hr. incubation, except for the reaction to the equilibrium mixture with triosephosphate. All the results are corrected for the known degree of hydrolysis of HDP and triosephosphate.

It will be noted that this reaction differs from the formation of CE in that it follows an almost linear course until the conversion is practically complete.

Table XIII

| Coenzymes | Amount of CE converted into EE (mg. P/5 ml.) 15 min. | 30 min. | 60 min. |
|---|---|---|---|
| A. Initial CE, 0·92 mg. P/5 ml. | | | |
| — | — | 0·02 | 0·03 |
| $MgCl_2$, *M*/250 | — | 0·12 | 0·10 |
| HDP, *M*/2000 | — | 0·07 | 0·12 |
| $MgCl_2$ + HDP | 0·23 | 0·42 | 0·79 |
| B. Initial CE, 0·96 mg. P/5 ml. | | | |
| — | — | — | 0·11 |
| $MgCl_2$, *M*/250 + HDP, *M*/2000 | — | — | 0·73 |
| ,, + HDP, *M*/2000 + ATP, *M*/10,000 | — | — | 0·77 |

*Concentration of $Mg^{++}$ and HDP.* The effect of varying the concentrations of $Mg^{++}$ and of HDP is shown in Table XIV. It is clear that $M/2000$ HDP is insufficient to give the maximal rate of reaction for between $M/4000$ and $M/2000$ the rate is proportional to the concentration, but higher concentrations were not used as this would have made the coenzyme concentration of the same order as the substrate concentration ($M/1000$ HDP=0·31 mg. P/5 ml.). The effect of changing the $Mg^{++}$ concentration will be mentioned again later.

Table XIV

Initial CE, 1·09 mg. P/5 ml.

HDP, $M/2000$. Incubated 60 min.

| Conc. of Mg ($M$) | 0 | $5\times10^{-4}$ | $10^{-3}$ | $2\times10^{-3}$ | $4\times10^{-3}$ | $10^{-2}$ |
|---|---|---|---|---|---|---|
| CE converted (mg. P) | 0·11 | 0·40 | 0·51 | 0·73 | 0·85 | 0·73 |

$MgCl_2$, $M/250$. Incubated 60 min.

| Conc. of HDP ($M$) | 0 | $2{\cdot}5\times10^{-5}$ | $5\times10^{-5}$ | $10^{-4}$ | $2{\cdot}5\times10^{-4}$ | $5\times10^{-4}$ |
|---|---|---|---|---|---|---|
| CE converted (mg. P) | 0·22 | 0·22 | 0·26 | 0·33 | 0·44 | 0·85 |

*Phosphoric acid β-glucoside*

Cori & Cori [1937] have shown that their ester is the α-glucoside of phosphoric acid. We attempted to make glucose-1-monophosphate synthetically by the method of Komatsu & Nodzu [1924], and obtained a product which differed in several ways from the natural ester. It was a glucose-1-monophosphate (total P 7·4 %, no reducing power before hydrolysis, phosphate and reducing sugar liberated simultaneously during hydrolysis), but it was more difficult to hydrolyse (62 % in 10 min., 84 % in 60 min. in $N$ HCl at 100°) and the rotation of its Ba salt was +22°. There is little doubt that this product was phosphoric acid β-glucoside, and the enzyme system in the presence of $Mg^{++}$ and HDP had no action on it.

*Experiments with glycogen*

In all the experiments with carbohydrate substrate described so far the substrate was starch. The essential results have been checked with glycogen in place of starch, and no qualitative difference has been found. The only quantitative difference consisted of a slightly greater rate of reaction with glycogen (Table XV).

Table XV

| Enzyme | Substrate | Coenzymes | Esters formed (mg. P/5 ml.) Total | CE | EE |
|---|---|---|---|---|---|
| GP3 | Starch | ATP, $M/2000$ | 0·70 | 0·59 | 0·11 |
| | | ,, +HDP, $M/2000$ | 1·24 | 0·39 | 0·85 |
| | Glycogen | ATP, $M/2000$ | 0·78 | 0·65 | 0·13 |
| | | ,, +HDP, $M/2000$ | 1·37 | 0·48 | 0·89 |
| GP4 | Starch | ATP, $M/2000$ | 0·61 | 0·57 | 0·04 |
| | | ,, +HDP, $M/2000$ | 0·68 | 0·41 | 0·45 |
| | Glycogen | ATP, $M/2000$ | 0·69 | 0·65 | 0·04 |
| | | ,, +HDP, $M/2000$ | 0·87 | 0·56 | 0·31 |

## Discussion

The position of inosinic acid as a coenzyme in the formation of CE is still obscure. Parnas & Mochnacka [1936] stated that IA is rather less active than AA, Cori & Cori [1937] that IA is one-half as active, and in our experiments IA

is only about one-tenth as active. (Parnas & Mochnacka do not state the concentration employed; Cori & Cori used AA at 0·1 mg. P/10 ml. (about $M/3000$) and presumably used IA at the same concentration; and we have used concentrations up to $M/1000$.) Our results might theoretically be explained in several ways. (1) In our preparations inosinic acid may be rapidly decomposed. This is extremely improbable on several grounds. (2) The resynthesis of AA from IA in the preparations of Parnas and Cori & Cori cannot be excluded *a priori*. On the other hand this reaction has never been observed in muscle preparations, and is again extremely improbable. (3) Yet another coenzyme may be required to enable IA to function in this reaction, and this substance may be present in the enzyme preparations of Parnas & Mochnacka, present at a lower concentration in the enzymes of Cori & Cori and almost absent from ours. This is a possible hypothesis, but we have no evidence as to the nature of this activator except that it is not HDP.

In our earlier experiments [Kendal & Stickland, 1937, 1] we showed that HDP is an essential coenzyme for glycolysis in our muscle extracts, and the present work shows one stage at which it is required (viz. CE → EE). The question arises as to the way in which the conversion of CE into EE is brought about in these muscle extracts which rapidly form EE with no coenzyme addition other than ATP or AA. In the experiments of Ostern *et al.* [1936] or Parnas & Mochnacka [1936] after 2–3 hr. incubation no ester but EE is found, and to achieve this result with our enzyme preparations the addition of HDP at a concentration of at least $M/2000$ would be required. It seems most unlikely that after very prolonged dialysis, autolysis followed by dialysis, or electrodialysis, the enzyme solutions used by these workers could contain HDP at a concentration of the order of $M/1000$, and this presents a difficult problem for which we can at present offer no solution. Moreover, the experiments of these workers were carried out at room temperature and under this condition we have found the conversion of CE into EE to take place much more slowly than at 37°.

The effect of the addition of Mg on the rates of the two reactions (starch → CE and CE → EE) also presents difficult problems. Cori & Cori [1937] found that with their dialysed enzyme preparations addition of $Mg^{++}$ had no effect on the former reaction, but a considerable accelerating effect on the latter, and conclude that, if $Mg^{++}$ is needed at all for the formation of CE, then a very low concentration is sufficient. Our experiments show clearly that $Mg^{++}$ is necessary in both reactions (Tables I and XIII), but the position is complicated by the following observations. (1) If the enzyme preparation is not free from $Mg^{++}$, so that esterification to CE takes place on addition of ATP alone, $Mg^{++}$ still has to be added to enable the second reaction to proceed. This is exactly in accordance with the results of Cori & Cori. (2) If the enzyme preparation is practically free from $Mg^{++}$, then the concentrations of $Mg^{++}$ required for activation are of the same order for each reaction (see Tables V and XIV). At present we can see no way of reconciling these two facts.

## Summary

A quantitative study has been made of what recent work suggests are the first two known steps in the formation of lactic acid from carbohydrate in muscle extracts, viz. (1) carbohydrate to glucose-1-monophosphate (Cori ester) and (2) Cori ester to hexose-6-monophosphate (Embden ester).

In (1) $Mg^{++}$ and adenosinetriphosphate or adenylic acid are essential, and inosinic acid is only slightly active as coenzyme. In (2) $Mg^{++}$ and hexose-

diphosphate are essential coenzymes, and the nucleotides play no part. In each case the effects of various factors, including the concentration of each of the coenzymes, on the rate of the reaction have been investigated.

We wish to thank Dr D. M. Needham for a great deal of advice.

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# LXXVI. THE MOLECULAR WEIGHTS OF PHYCOERYTHRIN AND PHYCOCYAN. I

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The molecular constants of phycoerythrin and phycocyan from various algae have previously been studied in this institute. Svedberg & Lewis [1928] found that at *p*H 6·8 the phycocyan from *Ceramium rubrum* of the Rhodophyceae family had half the molecular weight of the phycoerythrin from the same alga. Svedberg & Katsurai [1929] studied the phycoerythrin and phycocyan from another of the Rhodophyceae, *Porphyra tenera*, and the phycocyan from a species of the Cyanophyceae, *Aphanizomenon flos aquae*. This work indicated that at the isoelectric points the molecular weights of all three were about the same, but in more alkaline solutions the phycocyans seemed to dissociate into half-molecules. Svedberg & Eriksson [1932] determined the *p*H-stability diagram of *Ceramium-phycocyan*, and found that its sedimentation constant in the *p*H region around the isoelectric point was almost identical with that of phycoerythrin. In a more alkaline region it dropped to a value that might correspond to half-molecules. In view of the regularities lately found among the protein molecular weights it was considered of importance to determine accurately the molecular weights of phycoerythrin and phycocyan and learn more about the latter's dissociation.

These investigations were carried out on phycocyan and phycoerythrin from *Ceramium rubrum*. The proteins were prepared by the method described previously [Svedberg & Eriksson, 1932].

### *The* p*H-stability diagram and molecular weight of phycoerythrin*

The sedimentation constant of phycoerythrin was determined at various *p*H in the high-speed ultra-centrifuge. The results are summarized in Table I. The protein is stable and homogeneous in the *p*H region 3–10. The mean value of the sedimentation constant is 12·0.[1] Between 10·5 and 11·4 it is partly dissociated into molecules of lower molecular weight. At 11·4 all the material of normal molecular weight has vanished. The sedimentation constant of the dissociation product is about 2. A slight amount of an intermediate dissociation component with a sedimentation constant of about 5 has also been observed. Below *p*H 3 the protein aggregates into a high-molecular heterogeneous product.

The determinations in Table I were made immediately after mixing the solutions. The dissociation, however, increases with time, as may be seen from Table II. Before centrifuging the solutions were left for 2–3 days at room temperature.

Molecular weight determinations were made by means of sedimentation equilibrium runs. For the measurement of the concentration gradient the

[1] Sedimentation constants are expressed in units of $10^{-13}$ and diffusion constants in units of $10^{-7}$.

Table I. *Phycoerythrin*

Centrifugal force about 160,000 times gravity, in the last three runs 260,000, protein conc. about 0·1 %, thickness of column of solution 0·3–1·2 cm., source of light mercury lamp, plates Imperial process, metol-hydroquinone developer.

| Solvent | *p*H | Total *M* | Light filter | $s_{20}$ | | |
|---|---|---|---|---|---|---|
| HCl, NaCl | 2·17 | 0·16 | NiO | 20·1* | | Heterogeneous |
| ,, ,, | 3·27 | 0·18 | ,, | 12·5 | | |
| HOAc, NaOAc, NaCl | 3·6 | 0·22 | ,, | 12·2 | | |
| ,, ,, | 4·6 | 0·22 | Wratten 77 | 11·6 | | |
| HOAc, NaOAc | 4·6 | 0·20 | ,, | 12·2 | | |
| ,, ,, | 5·3 | 0·20 | NiO | 12·1 | | |
| $KH_2PO_4$, $Na_2HPO_4$, NaCl | 5·8 | 0·24 | ,, | 11·9 | | |
| ,, ,, ,, | 5·9 | 0·25 | ,, | 11·7 | | |
| ,, ,, ,, | 6·3 | 0·22 | ,, | 11·8 | | |
| ,, ,, ,, | 6·8 | 0·22 | ,, | 11·9 | | |
| ,, ,, ,, | 6·8 | 0·22 | $Cl_2$, $Br_2$ | 12·2 | | |
| ,, ,, ,, | 6·8 | 0·28 | Wratten 77 | 12·0 | | |
| ,, ,, ,, | 7·2 | 0·22 | NiO | 12·4 | | |
| ,, ,, ,, | 7·8 | 0·24 | ,, | 11·8 | | |
| $KH_2PO_4$, $Na_2B_4O_7$, NaCl | 8·3 | 0·25 | ,, | 12·1 | | |
| ,, ,, ,, | 8·6 | 0·25 | ,, | 12·1 | | |
| ,, ,, ,, | 9·00 | 0·20 | ,, | 11·6 | | |
| $Na_2CO_3$, $Na_2B_4O_7$, NaCl | 9·3 | 0·25 | ,, | 12·0 | | |
| ,, ,, ,, | 9·84 | 0·25 | ,, | 11·6 | | |
| ,, ,, ,, | 10·5 | 0·25 | ,, | 11·6 | + | |
| $Na_2HPO_4$, NaOH, NaCl | 11·0 | 0·25 | ,, | 11·2* | 3·29 | |
| ,, ,, ,, | 11·4 | 0·26 | ,, | | 1·88 | Heterogeneous |
| ,, ,, ,, | 11·7 | 0·22 | Wratten 77 | | 4·98, 2·0 | ,, |
| ,, ,, ,, | 11·9 | 0·22 | ,, | | 2·1 | ,, |
| | | | Mean | 12·0 | | |

* Not used for the calculation of mean value.

Table II. *Time effect on phycoerythrin at alkaline* p*H*

| Solvent | *p*H | Total *M* | Light filter | $s_{20}$ | |
|---|---|---|---|---|---|
| $Na_2B_4O_7$, $Na_2CO_3$, NaCl | 9·84 | 0·25 | NiO | 11·6 | + |
| ,, ,, ,, | 10·5 | 0·25 | ,, | 11·5 | 2·2 |
| $Na_2HPO_4$, NaOH, NaCl | 11·0 | 0·26 | ,, | 11·7 | + |
| ,, ,, ,, | 11·3 | 0·26 | ,, | | 5·48, 1·58 |
| ,, ,, ,, | 11·9 | 0·22 | Wratten 77 | | 4·68, 1·75 |

refractometric scale method was used. This procedure is more accurate than the earlier light absorption method, and allows a better control of the establishment of the equilibrium. Two determinations were made under the following conditions: protein concentration 0·22 %, *p*H 6·8 (0·01 *M* in $KH_2PO_4$ and $Na_2HPO_4$, 1 % NaCl), exposures made after 100–150 hr., speed 83 rev. per sec., temperature 293°, thickness of column of solution 0·4 cm., length 0·5 cm. The values obtained were 286,000 and 298,000, mean 293,000. The constancy of molecular weight demonstrates the homogeneity of the protein.

Tiselius & Gross [1934] obtained the value 4·00 for the diffusion constant of phycoerythrin and this combined with the sedimentation constant 12·0 gives the value 290,000 for the molecular weight.

*The pH-stability diagram and molecular weight of phycocyan*

Some sedimentation velocity runs were made at different *p*H. They showed good agreement with the stability diagram already published [Svedberg & Eriksson, 1932]. The values given in that paper, however, had not been corrected for the density and viscosity of the solvent. The protein is homogeneous with regard to molecular weight in the *p*H regions 2·5–6 and 7–8·5. The value of the sedimentation constant is 11·4 in the former and 6·2 in the latter region. (These figures include the corrected old as well as the new determinations.) Between *p*H 6 and 7 the protein consists of a mixture of both kinds of molecules. The results are summarized in Table III.

Table III. *Phycocyan*

Centrifugal force 100,000–200,000 times gravity, protein concentration about 0·1 %, thickness of column of solution 0·6–1·2 cm., light-filter nickel oxide glass, other conditions as in Table I.

| Solvent | *p*H | Total *M* | $s_{20}$ A | $s_{20}$ B |
|---|---|---|---|---|
| HCl, KCl | 2·0 | 0·05 | 10·6* | — |
| ,, ,, | 2·5 | 0·05 | 11·5 | — |
| ,, ,, | 3·0 | 0·04 | 11·5 | — |
| NaOAc, HOAc, NaCl | 3·5 | 0·22 | 11·0 | — |
| ,, ,, ,, | 3·6 | 0·24 | 11·3 | — |
| ,, ,, ,, | 3·9 | 0·24 | 11·3 | — |
| ,, ,, ,, | 4·0 | 0·22 | 11·7 | — |
| ,, ,, ,, | 4·0 | 0·24 | 11·3 | — |
| ,, ,, ,, | 4·5 | 0·28 | 11·7 | — |
| ,, ,, ,, | 5·0 | 0·22 | 12·3 | — |
| ,, ,, ,, | 5·0 | 0·22 | 11·9 | — |
| ,, ,, ,, | 5·0 | 0·22 | 11·5 | — |
| $KH_2PO_4$, $Na_2HPO_4$ | 5·5 | 0·10 | 11·1 | — |
| $KH_2PO_4$, $Na_2HPO_4$, NaCl | 5·5 | 0·22 | 11·6 | — |
| $KH_2PO_4$, $Na_2HPO_4$ | 5·8 | 0·10 | 11·1 | — |
| ,, ,, | 6·2 | 0·10 | 11·5 | 5·9 |
| ,, ,, | 6·2 | 0·10 | 11·5 | 5·5 |
| ,, ,, | 6·2 | 0·10 | 11·6 | 6·7 |
| $KH_2PO_4$, $Na_2HPO_4$, NaCl | 6·5 | 0·22 | 10·6* | 6·28 |
| ,, ,, ,, | 6·8 | 0·22 | 10·3* | 6·06 |
| $KH_2PO_4$, $Na_2HPO_4$ | 7·1 | 0·10 | — | 6·8 |
| $KH_2PO_4$, $Na_2HPO_4$, NaCl | 7·2 | 0·22 | — | 6·34 |
| $KH_2PO_4$, $Na_2HPO_4$ | 7·3 | 0·10 | — | 6·4 |
| $KH_2PO_4$, $Na_2HPO_4$, NaCl | 7·5 | 0·22 | — | 6·13 |
| $KH_2PO_4$, $Na_2HPO_4$ | 7·7 | 0·10 | — | 6·8 |
| $KH_2PO_4$, $Na_2HPO_4$, NaCl | 7·8 | 0·22 | — | 6·15 |
| $KH_2PO_4$, $Na_2HPO_4$ | 8·0 | 0·10 | — | 6·08 |
| $KH_2PO_4$, $Na_2B_4O_7$, NaCl | 8·0 | 0·20 | — | 6·3 |
| ,, ,, ,, | 8·5 | 0·20 | — | 6·00 |
| $Na_2HPO_4$ | 8·6 | 0·10 | — | 5·6* |
| $Na_2HPO_4$, NaOH, NaCl | 9·1 | 0·21 | — | 4·1* |
| | | Mean | 11·4 | 6·24 |

* Not used for the calculation of mean value.

Molecular weight determinations by means of sedimentation equilibrium measurements were made in both these regions. The data are summarized in Tables IV and V. The molecular weights found are 273,000 for the normal and 146,000 for the dissociated protein.

The diffusion constant for the undissociated phycocyan is 4·05 [Tiselius & Gross, 1934] and for the dissociation product 4·58 [A. G. Polson, unpublished]. Combined with the sedimentation constants these values give the molecular weight 272,000 for the undissociated and 131,000 for the dissociated molecules.

Table IV. *Undissociated phycocyan*

$p$H = 5·0 (0·02 *M* acetate, 0·2 *M* NaCl), speed 83·0 rev. per sec., cells 1·0 and 1·2 cm.

| Exp. no. | Molecular weight |
|---|---|
| 1 | 263,000 |
| 2 | 284,000 |
| 3 | 264,000 |
| 4 | 281,000 |
| Mean | 273,000 |

Table V. *Dissociated phycocyan*

$p$H = 7·5 (*M*/15 phosphate, 1 % NaCl); speed 130 rev. per sec.
other conditions as in Table IV

| Exp. no. | Molecular weight |
|---|---|
| 1 | 147,000 |
| 2 | 143,000 |
| 3 | 151,000 |
| 4 | 141,000 |
| Mean | 146,000 |

## Discussion

The molecular weights of phycocyan and phycoerythrin have been determined by means of two independent methods, giving within limits of experimental error the same results. The mean values for the molecular weights obtained are, in the $p$H regions around the isoelectric points, for phycoerythrin 291,000 and for phycocyan 273,000. In these regions, therefore, the proteins are of almost identical molecular weight. The molecular weight of the dissociated phycocyan is 138,000, which is half the normal value. This demonstrates that the phycocyan splits up into half-molecules.

Svedberg has advanced the hypothesis that all protein molecules are simple multiples of a common submolecule of molecular weight 17,600, obtained by dividing all the determined protein molecular weights by simple numbers [Svedberg, 1937]. In this system phycocyan and phycoerythrin belong to the group containing 16 submolecules, the calculated molecular weight for this group being 282,000. This group also contains the plant globulins amandin, excelsin and edestin. The dissociated phycocyan should contain 8 submolecules. No proteins with molecular weights falling between these two groups are known.

The discrepancy between these molecular weight values and those found by Lewis, viz. 208,000 for phycoerythrin and 106,000 for dissociated phycocyan may be due to the short time of his centrifuging. His calculations are made from the concentration distribution obtained after 40–50 hr., which, according to later experience, does not allow the establishment of complete equilibrium.

## Summary

1. The sedimentation constant of phycoerythrin has been investigated in the $p$H range 2–12. It is independent of $p$H from 3 to 10. Its mean value is 12·0 in this region.

2. The molecular weight of phycoerythrin has been determined. Two sedimentation equilibrium runs gave a mean of 292,000. By combining sedimentation and diffusion the value 290,000 was obtained.

3. The sedimentation constant of phycocyan has been recalculated and some new determinations made. For the undissociated molecule the value 11·4 was obtained and for the dissociated molecule 6·2.

4. The molecular weight of phycocyan has been determined. From sedimentation equilibrium measurements the values 273,000 for normal and 146,000 for dissociated phycocyan were found. A combination of sedimentation and diffusion gave 272,000 and 131,000 respectively.

5. The molecular weight determinations on phycocyan confirm the earlier conclusion that the molecules of this protein split into halves when they dissociate.

The author is indebted to Prof. Svedberg for suggesting this problem and for his kind interest during the work. The expenses connected with this investigation were defrayed by grants from the Andersson foundation and the Nobel foundation.

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# LXXVII. PHOSPHOESTERASES OF BONE AND SNAKE VENOMS

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(*Received 12 January 1938*)

In the course of experiments on the constitution of substances containing organically combined phosphorus in the form of phosphoric esters, it became necessary to obtain preparations of some of the different types of phosphoesterases [Kay, 1936] free from each other. Although many investigators have described the phosphoesterases obtained from a large and varied number of animal and vegetable tissues, it is believed that the present communication records hitherto untried sources which should be of value in this field.

## SECTION A

### Alkaline phosphomonoesterase

One of the most readily accessible sources of the "alkaline" type (Class A 1, liberating phosphoric acid from phenylphosphate, glycerophosphate, nucleotides [Kay, 1936]) of phosphomonoesterase is bone, and Martland & Robison [1929] have described a simple method of isolating the enzyme from the leg bones of young rabbits. It is, however, frequently difficult or costly to obtain such animals, and it is now shown that the metatarsal bones of sheep forelegs, which are slaughter-house offal, are suitable material for large-scale preparations. The material obtained from these bones by the Martland-Robison method contains both phosphomonoesterase and phosphodiesterase, and these may be separated by adsorption on norite charcoal, which adsorbs both enzymes. Elution with borate buffer at *p*H 8·6 liberates the phosphomonoesterase, but leaves the phosphodiesterase on the norite, from which we have as yet been unable to elute it. The same procedure has been used to purify the phosphomonoesterase of guinea-pig femurs. Arising out of these observations, a method is now described for the preparation of phosphomonoesterase from beef or sheep bones and the simultaneous elimination of phosphodiesterase by direct adsorption of the enzymes on norite charcoal from the crude autolysate without the alcohol-ether precipitation of the Martland-Robison method, which is costly and troublesome if carried out on a large scale.

### Experimental

*Estimations of phosphate and phenol.* All estimations of inorganic phosphate in this paper were made by Briggs's colorimetric method, and of phenol by the method of Folin & Denis [1915]. Every experiment was accompanied by a control without enzyme which was negative as regards liberation of phosphate or phenol respectively.

*Preparation.* The metatarsal bones of sheep forelegs or of guinea-pig femurs, obtained as soon as possible after death, were skinned, scraped free from tissue, sawn lengthwise or split, freed from marrow, crushed and allowed to autolyse

for 7–10 days in five times their weight of water containing chloroform. Methcol (2 vol.) and ether (3 vol.) were added to the filtered extract, and the resulting precipitate (A) was centrifuged and dried over phosphoric oxide. Less successful methods of precipitating active material were (i) adjustment of the reaction to *p*H 4·5, and (ii) adjustment to *p*H 4·5 followed by saturation with ammonium sulphate.

Table I records the activities of precipitate A from sheep bones: similar results were obtained with guinea-pig bones.

Table I

(*a*) Sodium β-glycerophosphate 3·8 mg.; precipitate A 20 mg.; *p*H 8·6 Clark and Lubs borate buffer 5 ml.; *M*/10 magnesium sulphate 0·5 ml. Diluted to 10 ml. with water. *p*H 8·6. 37°. 2 ml. samples.

(*b*) Yeast adenylic acid, or muscle adenylic acid, 5·1 mg., in dilute sodium carbonate, or sodium diphenylphosphate 6·0 mg.; precipitate A 30 mg.; *p*H 8·6 borate buffer 5 m.; *M*/10 magnesium sulphate 0·5 ml. Diluted to 10 ml. with water. *p*H 8·6. 2 ml. samples.

| Time hr. | Glycero-phosphate hydrol. % | Yeast adenylic hydrol. % | Muscle adenylic hydrol. % | Diphenyl-phosphate hydrol. % |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 2 | 49 | 44 | 49 | 15 |
| 4 | — | 60 | 76 | — |
| 7 | 49 | — | — | 21 |
| 8 | — | 73 | 77 | — |
| 16 | — | — | — | 23 |

*Dialysis of precipitate A.* An aqueous suspension was dialysed through No. 400 cellophane against distilled water until free from inorganic phosphate. The enzyme was then precipitated by addition of methcol-ether (2 : 3), centrifuged and dried over phosphoric oxide. This treatment resulted in a considerable increase in hydrolytic activity as shown by Table II.

Table II

Yeast adenylic acid or muscle adenylic acid 5·6 mg.; dialysed enzyme preparation 30 mg.; *p*H 8·6 borate buffer 5 ml.; *M*/10 magnesium sulphate 0·5 ml. Diluted to 10 ml. with water, *p*H 8·6. 37°. 2 ml. samples.

| Time hr. | Yeast adenylic hydrol. % | Muscle adenylic hydrol. % |
|---|---|---|
| 0 | — | — |
| 2 | 78 | 78 |
| 4 | 110 | — |
| 6 | 110 | 106 |

*Purification by adsorption of monoesterase in precipitate A from sheep or guinea-pig bones*

The norite charcoal used in these experiments had been extracted successively with the following hot solvents—20 % HCl, alcohol and glacial acetic acid—being washed thoroughly with much distilled water between each extraction, and had then been dried at 110°. A mixture of charcoal (800 mg.) and a finely ground suspension of precipitate A (300 mg.) in water (10 ml.) was shaken occasionally for 1 hr. at 0°, and the charcoal was collected, washed with water and eluted overnight with *p*H 8·6 borate buffer (12 ml.). This solution contained phosphomonoesterase, but no phosphodiesterase, as shown in the

case of sheep bone preparations in Table III. It showed no differentiation in the rates of hydrolysis of purine and pyrimidine nucleotides or of adenosine-3- and 5-phosphates. Similar results were obtained with guinea-pig bone preparations.

Table III

(*a*) Sodium β-glycerophosphate 3·76 mg. or sodium diphenylphosphate 5 mg.; enzyme solution 2 ml.; *p*H 8·6 borate buffer 5 ml.; *M*/10 magnesium sulphate 0·5 ml. Diluted to 10 ml. with water. *p*H 8·6. 37°. 2 ml. samples.

(*b*) Yeast adenylic acid, muscle adenylic acid or cytidylic acid, 14·5 mg. in dilute sodium carbonate; enzyme solution 2 ml.; *p*H 8·6 borate buffer 10 ml.; *M*/10 magnesium sulphate 1·0 ml. Diluted to 20 ml. with water. *p*H 8·6. 37°. 2 ml. samples.

| Time hr. | Glycero-phosphate hydrol. % | Diphenyl-phosphate hydrol. % | Yeast adenylic hydrol. % | Muscle adenylic hydrol. % | Cytidylic hydrol. % |
|---|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 41 | 0 | 14 | 10 | 10 |
| 4 | 57 | 0 | 25 | 25 | 13 |
| 6 | — | 0 | 37 | 29 | 23 |
| 24 | 62 | 0 | 64 | 64 | 60 |

The *p*H optimum of the purified phosphomonoesterase of sheep bone, using β-glycerophosphate as substrate, was at *p*H 8·8 (Fig. 1).

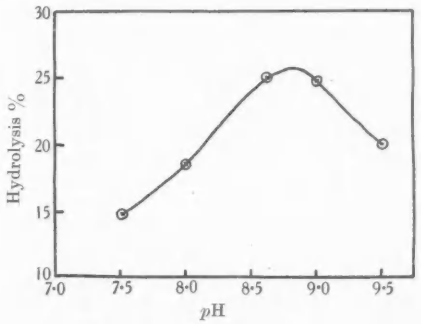


Fig. 1. Sodium β-glycerophosphate 3·03 mg. in water 2 ml.; enzyme solution 2 ml.; borate or veronal buffer 5 ml.; *M*/10 magnesium sulphate 0·5 ml. Diluted to 10 ml. with water. 3 hr. 37°. 4 ml. samples.

*Attempts to obtain the phosphodiesterase of sheep bone.* The following procedures were unsuccessful:

(i) Preservation of suspension of precipitate A at *p*H 4·5 for 2 days in the hope of inactivating the monoesterase. No appreciable inactivation of either enzyme occurred.

(ii) After the monoesterase had been eluted from the charcoal, elution with dilute acetic acid yielded a solution which had no diesterase activity towards diphenylphosphate at *p*H 8·6.

(iii) After elution of the monoesterase, elution with *p*H 7 phosphate buffer or *M*/5 potassium dihydrogen phosphate, followed by dialysis to remove phosphate, failed to liberate diesterase.

*Simultaneous preparation and purification of bone phosphomonoesterase by adsorption without alcohol-ether precipitation*

Beef leg-bones, not necessarily obtained immediately after the death of the animal, were freed from marrow, sawn and hashed in pieces of about the size of a walnut or smaller, and autolysed for 7 days with four times their weight of water containing chloroform. The phosphoesterases in this autolysate (400 ml.) were adsorbed on purified norite charcoal (3 g.) for 1–1·5 hr. at 0°, and the charcoal was collected, washed with water and eluted with *p*H 8·6 borate buffer (20 ml.).

A further adsorption of the filtrate (200 ml.) from the first adsorption was carried out by means of norite (1 g.) for 1 hr. at 0°, followed by washing with water and elution of the norite with *p*H 8·6 borate buffer (10 ml.).

The above elutions contained monoesterase but not diesterase, and analogous experiments with the autolysates from sheep metatarsals had similar results (Table IV). The procedure described above has the additional advantage that inorganic phosphate is automatically removed from the enzyme preparation, since it remains in the filtrates.

Table IV

(*a*) Sodium β-glycerophosphate 3·62 mg.; enzyme solution from 1st or 2nd adsorption 4 ml.; *p*H 8·6 borate buffer 5 ml.; *M*/10 magnesium sulphate solution 0·5 ml. Diluted to 12 ml. with water. 2 ml. samples.

(*b*) Sodium diphenylphosphate 6·7 mg.; enzyme solution 4 ml.; *p*H 8·6 borate buffer 5 ml.; *M*/10 magnesium sulphate 0·5 ml. Diluted to 20 ml. with water. 37°. 4 ml. samples.

| | Beef | | | Sheep | |
|---|---|---|---|---|---|
| | 1st adsorption | | 2nd adsorption | 1st adsorption | |
| Time hr. from zero | Glycero-phosphate hydrol. % | Diphenyl-phosphate hydrol. % | Glycero-phosphate hydrol. % | Glycero-phosphate hydrol. % | Diphenyl-phosphate hydrol. % |
| 4 | 70 | 0 | 32 | 40 | 0 |
| 24 | 72 | 0 | — | 67 | 0 |

## SECTION B

### Phosphodiesterases

Many animal and vegetable tissues contain both phosphomonoesterases and phosphodiesterases (liberating phenol, but not phosphoric acid, from diphenylphosphate), but despite the efforts of various investigators there is no recorded instance in which a diesterase preparation, free from monoesterase, has been obtained from such sources by removal of the monoesterase. In the present work, for example, all attempts failed to obtain the diesterase of sheep bone from the charcoal adsorbate of this enzyme and phosphomonoesterase. These results find parallels in the lack of success of Uzawa [1932] in eluting from adsorbents the diesterases of a variety of animal and vegetable sources. The same lack of success is apparent if one assumes—as seems to be justified—that the polynucleotidases, which split nucleic acids into their constituent nucleotides, are wholly or in part diesterases. Thus, for example, Levene & Dillon [1932] and Makino [1934] obtained from intestinal mucosa preparations of mixed polynucleotidase and nucleotidase, the latter enzyme being a phosphomonoesterase in the terms of this classification. Levene & Dillon were unable to separate these enzymes, but Makino was apparently able to use the mixture to prepare both nucleotides and nucleosides.

The nearest approach to the production of an enzyme preparation having diesterase, but no monoesterase, activity is to be found in the work of Klein [1933], who obtained from calf's intestinal mucosa a mixture of thymonucleinase, a polynucleotidase specific for thymus nucleic acid, and of a phosphomonoesterase which eliminated phosphoric acid from the resulting nucleotides. He was able to suppress the action of the phosphomonoesterase by means of sodium arsenate without interfering with the activity of the thymonucleinase.

Uzawa [1932], however, found in the venom of a Japanese snake known as the Habu (*Trimeresurus flavoviridis*) a diesterase entirely free from monoesterase, and he also stated that the venom of the Mamushi (*Agkistrodon blomhoffi*) also contains phosphodiesterase, without referring to the presence, or absence, of monoesterase. Klein & Rossi [1935], on the other hand, found both diesterase and monoesterase in a specimen of Habu venom obtained from Japan, and were moreover unable to separate these enzymes.

## Table V

Monophenylphosphate or diphenylphosphate, 13 mg., in dilute sodium carbonate; venom 20 mg., *p*H 8·6 borate buffer 10 ml. Diluted to 20 ml. with water and incubated at 37°. 2 ml. samples. In the case of monoesterase the degree of hydrolysis was calculated from estimations of inorganic phosphate, in the case of diesterase from estimations of phenol. When a monoesterase accompanied the diesterase, theory demanded the liberation of two phenol molecules from one of diphenylphosphate, but when no monoesterase was present only one molecule of phenol should be liberated. The figures for percentage hydrolysis are calculated on this basis.

| | | | Monophenyl-phosphate | | Diphenyl-phosphate | |
|---|---|---|---|---|---|---|
| Snake | Habitat and Family | Venom collected | Time hr. | Hydrol. % of theory | Time hr. | Hydrol. % of theory |
| *Notechis scutatus* Tiger snake | Australia Colubridae | 1931–34 mixed | 6 | 9·5 | 24 | 9·1 |
| *Bungarus fasciatus* Banded krait | South-east Asia Colubridae | May 1937 | 24 | 0 | 24 | 41·6 |
| *Naja haje* Egyptian cobra | North and Central Africa Colubridae | Jan. 1936 | 6 | 59·0 | 24 | 29·0 |
| *Naja Naja* Black spitting var. of Indian cobra | Malaya Colubridae | 1936 | 3 | 64·0 | 24 | 33·0 |
| *Sepedon haemachata* Ringhals | South Africa Colubridae | Oct. 1935 | 6 | 22·9 | 5 | 21·6 |
| *Vipera russellii* Russell's viper | India, etc. Viperidae | Aug. 1937 | 24 | 0 | 24 | 41·8 |
| *Bitis arietans* Puff-adder | South Africa Viperidae | Nov. 1936 | 24 | 58·0 | 24 | 14·9 |
| *Agkistrodon piscivorus* Water mocassin | Southern U.S.A. Crotalidae | May 1937 | 24 | 0 | 24 | 20·4 |
| *Agkistrodon mokasin* Copperhead | Eastern U.S.A. Crotalidae | 1935 | 24 | 0 | 24 | 13·9 |
| *Trimeresurus Wagleri* Wagler's pit viper | Malaya Crotalidae | May 1935 | 2 | 64·3 | 24 | 17·5 |
| *Crotalus oreganus* Oregon rattlesnake | Western U.S.A. Crotalidae | May 1935 | 24 | 0 | 24 | 24·6 |
| *Crotalus adamanteus* Diamond rattlesnake | Southern U.S.A. Crotalidae | July 1936 | 24 | 0 | 24 | 45·0 |

These discrepancies, the need of finding a comparatively accessible source of monoesterase-free diesterase, and a desire to enquire whether the enzyme content varies with the snake family, prompted us to examine the venoms of snakes as regards their "alkaline" monoesterase and their diesterase activities, and we have been able to do this through the generosity of Dr Burgess Barnett, to whom we acknowledge with pleasure our indebtedness and gratitude for advice and for supplying the venoms. The venoms were extracted from living snakes and were dried *in vacuo* at 35° immediately after extraction. They were then hermetically sealed and stored in the dark. The results are tabulated in Table V.

The substrates examined were monophenylphosphate (monoesterase) and diphenylphosphate (diesterase); the actions of the venoms on other simple substrates such as α- and β-glycerophosphates have not yet been studied. It was necessary to use the same substrates throughout so as to obtain comparative results, and this restriction was also enforced because the amount of venom available in several cases was small and the material difficult to procure.

Several venoms contained diesterase but not monoesterase, whereas none contained monoesterase but no diesterase. In connexion with the work of Uzawa and of Klein & Rossi, mentioned above, it may be observed that *Trimeresurus wagleri* venom contained both mono- and di-esterases and that the venoms of *Agkistrodon piscivorus* and *A. mokasin* contained diesterase but not monoesterase.

Apparently the snake family—Crotalidae, Colubridae, Viperidae—is not a deciding factor in determining which of the phosphoesterases are present in the venom.

The *p*H-activity curve was determined for the diesterase in the venom of Russell's viper (Fig. 2).

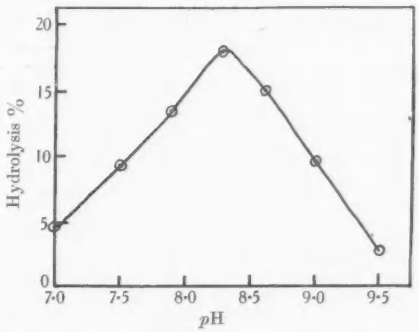


Fig. 2. Sodium diphenylphosphate 3·08 mg. in water 2 ml.; Russell's viper venom 7 mg.; borate or veronal buffer 5 ml.; diluted to 10 ml. with water and incubated at 37° for 3 hr. 4 ml. samples.

## Summary

1. The phosphomonoesterase (optimum *p*H 8·8, sheep) and the phosphodiesterases of sheep, beef and guinea-pig bones are adsorbed by norite charcoal from aqueous solution. Since the diesterase is not eluted, the monoesterase, which can be eluted, is purified from diesterase and inorganic phosphate.

2. Such adsorptions may be carried out with the solution of an enzyme preparation obtained by the customary precipitation with alcohol and ether, or

may be used with a crude bone autolysate, thus obviating this precipitation completely.

3. Of the venoms of twelve different snakes of the viperidae, crotalidae and colubridae, seven contained both phosphomonoesterase and phosphodiesterase, whereas five contained phosphodiesterase only, optimum at *p*H 8·3 in the case of Russell's viper venom.

4. Apparently the snake family is not a deciding factor in determining which phosphoesterases are present in the venom.

We wish to express our gratitude to the Government Grant Committee of the Royal Society for a research grant.

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# LXXVIII. 5-NUCLEOTIDASE

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(*Received 23 January 1938*)

Reis [1934] described an enzyme, called by him 5-nucleotidase, which occurs in the brain of the rabbit and rat and in the retina of the frog, rat, rabbit and calf. Extracts of these tissues dephosphorylated muscle adenylic acid (adenosine-5-phosphate) and inosinic acid (inosine-5-phosphate) many times more rapidly than yeast adenylic acid (adenosine-3-phosphate) or glycerophosphate.

Subsequently, Reis [1937] showed the presence of the same enzyme in calf brain and horse nerve, and extended the list of substances which are only slowly dephosphorylated to include Embden's hexosemonophosphate, pyrophosphate, phosphoglyceric acid, phosphogluconic acid and guanine nucleotide. He attributed this unspecific hydrolysis to the action of a general phosphatase present in small amount in nervous tissue, whereas the rapid fission of adenosine (inosine)-5-phosphate is caused by 5-nucleotidase.

The position of Harden & Young's fructose-1:6-diphosphate was intermediate between that of adenosine-5-phosphate and that of the group of slightly reactive substances. Reis attributed this to the presence of the furanose ring in fructose-1:6-diphosphate, a structure also found in adenosine-5-phosphate but not in Embden's hexosemonophosphate, which is a pyranose.

In connexion with the present investigations, it should be noted that the duration of the experiments of Reis did not exceed 5 hr.

## Experimental

Taking into consideration only the first few hours of the experiments of the present researches, extracts of calf retina and sheep retina resembled those described by Reis; they dephosphorylated adenosine-5-phosphate rapidly and

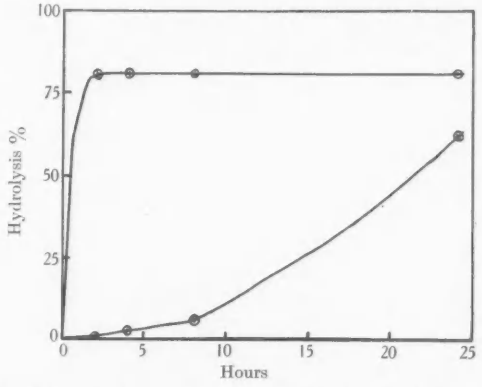


Fig. 1. Dephosphorylation of adenosine-5-phosphate and adenosine-3-phosphate by extract of calf retina under similar conditions.

had but little action on adenosine-3-phosphate. After this preliminary period, however, the rate of hydrolysis of adenosine-3-phosphate increased markedly, and at the end of 22–24 hr. the degrees of hydrolysis of the two esters were the same (Fig. 1).

The enzyme solutions were prepared from the retinae, obtained as soon as possible after death, by grinding with clean sand and chloroform-water (1 ml.: each retina) and allowing the mixture to autolyse for 24 hr. at room temperature before filtration. The filtrate is referred to as the enzyme solution. Estimations of phosphate were carried out by the Briggs's colorimetric method.

Table I. *Typical enzymic activities of extracts of calf retina and sheep retina*

(*a*) *Calf.* Yeast or muscle adenylic acid 4·2 mg., dissolved in dilute sodium carbonate; *p*H 7 veronal buffer 5 ml.; enzyme solution 2 ml.; water to 10 ml., with a drop of toluene. 37°. 2 ml. samples.

(*b*) *Sheep.* Yeast or muscle adenylic acid 5·1 mg., dissolved in dilute sodium carbonate; *p*H 7 veronal buffer 5 ml.; enzyme solution 3 ml.; water to 10 ml., with toluene. 37°. 2 ml. samples.

| | Calf retina | | Sheep retina | |
|---|---|---|---|---|
| Time hr. | Adenosine 5-phosphate hydrol. % | Adenosine 3-phosphate hydrol. % | Adenosine 5-phosphate hydrol. % | Adenosine 3-phosphate hydrol. % |
| 0 | — | — | — | — |
| 2 | 81 | 1·8 | 61 | 5·5 |
| 4 | 81 | 3·9 | — | — |
| 5 | — | — | 64 | 44 |
| 8 | 81 | 6·5 | — | — |
| 22 | — | — | 100 | 100 |
| 24 | 81 | 62 | — | — |

The specific action on adenosine-5-phosphate vanished when the enzyme solutions were dialysed through No. 400 cellophane against distilled water until free from inorganic phosphate, both the 3- and 5-nucleotides being then dephosphorylated immediately and at similar rates (Table II).

Table II. *Enzymic activities of dialysed extract of sheep retina*

Yeast or muscle adenylic acid 4·6 mg., in dilute sodium carbonate; *p*H 7 veronal buffer 5 ml.; dialysed enzyme solution 3 ml.; water to 10 ml. and toluene. 37°. 2 ml. samples.

| Time hr. | Adenosine-3-phosphate hydrol. % | Adenosine-5-phosphate hydrol. % |
|---|---|---|
| 0 | — | — |
| 2 | 55 | 73 |
| 4 | 76 | 79 |
| 8 | 85 | 85 |

The foregoing results suggested that retina extracts contained two enzymes, 5-nucleotidase and a phosphomonoesterase capable of dephosphorylating adenosine-3-phosphate. Attempts to purify or separate the two enzymes by adsorption on norite charcoal and elution with *p*H 7·8 borate buffer or *p*H 6·0 acetate buffer were unsuccessful; the enzymes were adsorbed but were not eluted, since the eluates had no action on yeast or muscle adenylic acids.

It was evident that the tissues discussed above could not in all probability be satisfactorily used for 5-nucleotidase preparations, and it was therefore decided to investigate other possible sources of this enzyme.

Recently, it was shown [Gulland & Jackson, 1938] that the venoms of Russell's viper (*Vipera russellii*), the water mocassin (*Agkistrodon piscivorus*), the banded krait (*Bungarus fasciatus*) and the diamond rattlesnake (*Crotalus adamanteus*) contain a diesterase, which liberates phenol from diphenylphosphate, but do not contain a monoesterase which can liberate inorganic phosphate from monophenylphosphate. It has now been observed that these venoms rapidly dephosphorylate adenosine-5-phosphate and inosine-5-phosphate [Embden, 1929] (Table III) but have no action whatsoever on adenosine-3-phosphate or α- or β-glycerophosphate. Further, it has been shown that Russell's viper venom entirely fails to liberate phosphate from guanylic acid, cytidylic acid, glucose-6-phosphate, fructose-6-phosphate, mannose-6-phosphate, trehalose monophosphate and fructose-1:6-diphosphate.

Table III. *Enzymic activities of the venoms of Russell's viper, water mocassin, banded krait and diamond rattlesnake*

Adenosine-5-phosphate 7·0 mg. in a little dilute sodium carbonate, or barium inosinate 7·0 mg., converted into sodium salt by means of sodium sulphate; 10 mg. venom* dispersed by grinding in water; *p*H 8·6 borate buffer 5 ml.; water to 10 ml. and some chloroform. 37°. 2 ml. samples.

| | Russell's viper 5-phosphate of | | Water mocassin 5-phosphate of | | Banded krait Adenosine-5-phosphate | Diamond rattlesnake Adenosine-5-phosphate |
|---|---|---|---|---|---|---|
| Time hr. | Adenosine hydrol. % | Inosine hydrol. % | Adenosine hydrol. % | Inosine hydrol. % | hydrol. % | hydrol. % |
| 0 | 22·0† | 30† | 32† | 25† | 9† | 18† |
| 2 | 74 | 58 | 70 | 58 | — | — |
| 3 | — | — | — | — | 64 | 70 |
| 4 | — | 61 | — | 63 | — | — |
| 6 | 76 | — | 70 | — | — | — |
| 24 | 79 | — | 75 | — | 70 | 72 |

These venoms did not liberate inorganic phosphate in 24 hr. from any of the other substrates mentioned in the text.

* The venoms, extracted from living snakes, were dried in a vacuum at 35° immediately after extraction. They were then hermetically sealed and stored in the dark.

† Since neither the venoms nor the substrates contained inorganic phosphate, this hydrolysis had occurred before the sample could be taken.

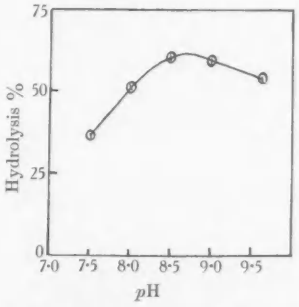


Fig. 2. *p*H-activity curve of 5-nucleotidase in Russell's viper venom. Adenosine-5-phosphate 1·51 mg. in 2 ml. water; venom 3 mg. dispersed in 2 ml. water; veronal or borate buffer 5 ml.; water to 10 ml. 37°. Time 30 min. 4 ml. samples.

It is evident that the venoms of Russell's viper, water mocassin, banded krait and diamond rattlesnake are rich in 5-nucleotidase, and in the first case

the enzyme is active over a comparatively wide *p*H range, with a slight optimum at *p*H 8·5–9 (Fig. 2).

## Discussion

The conclusions drawn by Reis from his experiments with brain, nerve and retina were that these tissues contain an enzyme, 5-nucleotidase, which is specific for adenosine(inosine)-5-phosphate, and that this enzyme is accompanied by small amounts of a non-specific phosphomonoesterase which, on account of its low concentration, effects only slight hydrolysis of other phosphoric esters, one of which is adenosine-3-phosphate. In the present investigation, these results have been confirmed as regards retinae when the experiments were of relatively short duration. It has now been shown, however, that if the experiments are more prolonged dephosphorylation of adenosine-3-phosphate occurs rapidly after an initial period of but slight hydrolysis. Moreover, it has been found that the degree of hydrolysis of adenosine-3-phosphate reaches that of adenosine-5-phosphate. The explanations put forward by Reis for the facts observed by him and hitherto sufficient now seem to require modification in the light of the new results, and in retinae there seems to be, at any rate potentially, a considerable amount of an enzyme which can dephosphorylate adenosine-3-phosphate.

The reasons for the time-lag in the hydrolysis of adenosine-3-phosphate are not clear. The shape of the hydrolysis curve makes it improbable that the lag is the effect of two existing enzymes acting in conjunction and causing, for example, a transfer of the phosphoric acid residue from the 3- to the 5-position followed by hydrolysis by the 5-nucleotidase, a change which would be analogous to the enzymic conversion of 3-phosphoglyceric acid into pyruvic acid by way of 2-phosphoglyceric acid [Lohmann & Meyerhof, 1934; Meyerhof & Kiessling, 1935]. The lag may be due to a delayed development or activation of the non-specific phosphomonoesterase which hydrolyses adenosine-3-phosphate, or to the development of an activator which enables 5-nucleotidase to attack this substrate. Or, finally, the lag may be the result of the gradual inactivation of an inhibitor which restrains the action of an already existing phosphomonoesterase. This last alternative may receive some experimental support from the fact that dialysis of the retina extract so changes its specificity that hydrolyses of both adenosine-3- and -5-phosphates occur immediately and at similar rates; possibly the inhibitor passes out through the dialysing membrane.

It is evident that the venoms of Russell's viper, the water mocassin, the banded krait and the diamond rattlesnake are less contaminated sources of 5-nucleotidase than brain, retina and nerve; these venoms very actively dephosphorylate adenosine-5-phosphate and inosine-5-phosphate, and although they contain a diesterase, they are entirely free from a non-specific phosphomonoesterase; one of them has no action on any of the eleven other phosphoric esters which were investigated, whilst the others behave similarly but have not been so fully investigated.

Reis observed that preparations from brain and nerve had considerable dephosphorylating action on fructose-1:6-diphosphate and attributed this hydrolysis to the presence of the furanose structure in this ester. Since the venom of Russell's viper has no dephosphorylating action whatsoever on fructose-1:6-diphosphate, it seems more probable that the hydrolysis observed by Reis was the result either of the action of a specific enzyme or of an increased susceptibility of this ester to the action of the non-specific phosphomonoesterase as compared with that of the group of other phosphoric esters which were but slightly attacked.

It should be noticed that the venoms which have been tested for 5-nucleotidase activity have all been found to contain this enzyme. Unfortunately it has not been possible to examine for its presence those venoms which contain a non-specific phosphomonoesterase [Gulland & Jackson, 1938], because the dephosphorylating action of the non-specific enzyme would mask the effect of the specific esterase. It is hard, however, to avoid the speculation that the presence of the active 5-nucleotidase, with its specific effect on two nucleotides which play important parts in vital processes, may be related to some of the toxic effects of snake venoms.

SUMMARY

1. Extracts of calf and sheep retinae contain specific 5-nucleotidase, and during the early part of experiments rapidly dephosphorylate adenosine-5-phosphate but have only slight action on adenosine-3-phosphate.

2. After an initial period in which the above conditions hold, dephosphorylation of adenosine-3-phosphate occurs rapidly, and soon the degrees of hydrolysis of the 3- and 5-nucleotides become similar.

3. Dialysis so alters retina extract that the specificity for adenosine-5-phosphate is lost and both 3- and 5-nucleotides are rapidly dephosphorylated immediately and at similar rates.

4. It is concluded that retina extract contains considerable amounts of a phosphomonoesterase which, under suitable conditions, can dephosphorylate adenosine-3-phosphate. This view is contrary to the conclusion of Reis.

5. The venoms of Russell's viper, the water mocassin, the banded krait and the diamond rattlesnake, are rich and specific sources of 5-nucleotidase, since they rapidly dephosphorylate adenosine-5-phosphate and inosine-5-phosphate but have no action whatsoever on monophenylphosphate, α- or β-glycerophosphate and adenosine-3-phosphate. Russell's viper venom also failed completely to dephosphorylate seven other monophosphoric esters.

6. Taking into consideration the experiments of Reis, 5-nucleotidase dephosphorylates specifically adenosine-5-phosphate and inosine-5-phosphate but does not attack fifteen other monophosphoric esters.

We acknowledge with pleasure our indebtedness to Dr Burgess Barnett for snake venoms, to Prof. R. Robison, for glucose-, fructose-, mannose- and trehalose-phosphates, and fructosediphosphate, and to the Government Grant Committee of the Royal Society for a research grant.

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# LXXIX. THE METABOLISM OF CREATINE. IV

## A. IMPROVED DETERMINATION OF GLYCOCYAMINE

## B. IMPROVED DETERMINATION OF ARGININE

By HORACE WILLARD DAVENPORT,
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(*Received 1 February 1938*)

## A. IMPROVED DETERMINATION OF GLYCOCYAMINE

By H. W. DAVENPORT and R. B. FISHER

THE technique for the determination of glycocyamine in tissue extracts by Weber's method [1935], as described by Bodansky [1936], appears to possess two major disadvantages. In the first place, the tissue or extract taken for analysis has to contain approximately 1 mg. of glycocyamine, although the amount of glycocyamine needed for the final colorimetric measurement is only 0·025 mg. In the second place, Bodansky [1936] was able to recover only $75 \pm 5$ % of added glycocyamine.

We have been able (method A below) to reduce the amount of glycocyamine needed for a determination to as little as 0·1 mg. and to recover $90 \pm 0{\cdot}6$ % of added glycocyamine. A simpler method (method B) is also described, which can be used in certain restricted circumstances, which recovers 100 % of added glycocyamine.

*Method A*

Reagents required:

10 % metaphosphoric acid.

Cold saturated baryta, and powdered barium hydroxide.

Fuller's earth. (B.D.H. "fuller's earth, levigated powder" is kept overnight in *N* HCl—200 ml. per 100 g., and then washed with water by decantation until the washings are Cl-free. The fuller's earth is filtered off by suction, dried on a water bath and ground until it passes a 90-mesh sieve.)

$5N$ $H_2SO_4$.

Basic lead carbonate ("white lead powder").

Permutite. (We have found a white variety supplied by Harrington Bros. to be very satisfactory after washing with water to remove the finer particles.)

0·04 % α-naphthol (stock solution of 0·2 % in absolute alcohol, diluted with water).

10 % NaOH.

40 % urea solution.

Sodium hypobromite solution (2 g. Br in 100 ml. 5 % NaOH).

The tissue to be analysed is minced finely under metaphosphoric acid, the mixture centrifuged and the supernatant liquid poured off. The residue in the tube is ground with a stout glass rod and extracted with more metaphosphoric

acid. After centrifuging and pouring off the extract, six further extractions are made, and the combined extracts are made up to an appropriate volume. In the instance of perfusates and other samples containing little protein, the protein is precipitated by adding enough metaphosphoric acid to give a final concentration of approximately 2%, the precipitate is removed by centrifuging and an aliquot of the supernatant fluid is taken for analysis.

Bodansky's [1936] procedure of autoclaving the tissue with HCl results in the liberation of large amounts of arginine from the tissue proteins, and arginine in large amounts interferes with the analysis. Metaphosphoric acid is preferred to trichloroacetic acid as a protein precipitant because trichloroacetic acid interferes with the colorimetric procedure and cannot readily be removed. Metaphosphoric acid can be removed as the Ba salt, as described below.

A measured volume of the combined extracts is placed in a centrifuge tube and 2 vol. of cold saturated baryta are added (where the concentration of glycocyamine is low, powdered $Ba(OH)_2$ is stirred in at 40° until the supernatant liquid gives no precipitate on the addition of one drop of cold saturated baryta). The barium metaphosphate is centrifuged off and an aliquot of the supernatant liquid is transferred to a volumetric flask (e.g. 10 ml. extract taken, 20 ml. baryta added, 20 ml. supernatant transferred to 25 ml. flask). $5N$ $H_2SO_4$ is added until the contents of the flask are acid to Congo red, and thereafter to make the final concentration $1{\cdot}2N$. The contents of the flask are made up to volume and the $BaSO_4$ separated by centrifuging. A sample of the solution containing 0·1–0·5 mg. glycocyamine is placed in a 25 ml. centrifuge tube together with sufficient water to make the final volume 5 ml., 1 g. of the prepared fuller's earth is added and the tube is shaken for 10 min. in the apparatus of Fisher & Wilhelmi [1938]. The fuller's earth is centrifuged down and the supernatant liquid discarded. Six glass beads (5 mm. diam.) are placed in the tube, 5 ml. water are added and the fuller's earth is stirred up with a pointed glass rod. The tube is then shaken in the apparatus until the fuller's earth is uniformly suspended.

The fuller's earth forms a very compact sticky mass after centrifuging, and the above technique is essential for its resuspension at this and later stages of the determination.

The fuller's earth is centrifuged down again and the washings discarded. It is resuspended in 5 ml. water and saturated baryta is added, 0·5 ml. at a time, until the solution is just alkaline to Congo red, the tube being shaken in the apparatus between additions of baryta.

This is a crucial stage in the procedure. $BaSO_4$ is precipitated when baryta is added to the fuller's earth suspension, and we have been able to show that $BaSO_4$ adsorbs glycocyamine in the presence of any appreciable concentration of free $Ba(OH)_2$. That is, an apolar adsorption appears to occur, of the type described by Phelps & Peters [1929] and Phelps [1929; 1931]. Since it also appears that glycocyamine adsorbed on $BaSO_4$ undergoes fairly rapid destruction, it is very important that the elution of glycocyamine from the fuller's earth should take place in the presence of a minimal excess of baryta. Bodansky [1936] elutes by grinding the fuller's earth with solid baryta, and we think it very probable that this procedure is responsible for his low recovery of glycocyamine.

The fuller's earth is kept suspended in the solution for 10 min. and is then centrifuged down. The eluate is transferred to a 25 ml. volumetric flask, and two further elutions are performed, the fuller's earth being suspended in 5 ml. of water for 10 min. in each instance. $5N$ $H_2SO_4$ is then added to the combined eluates until the solution is acid to Congo red (one drop should suffice). The contents of the flask are then made up to volume and approximately 10 ml. are

poured into a 15 ml. centrifuge tube containing about 1 g. of basic lead carbonate. The tube is shaken until the contents are alkaline to Congo red and then 0·4 g. permutite is added and the tube shaken for 10 min., preferably by turning over and over slowly. The contents are centrifuged and the supernatant liquid is transferred to another 15 ml. centrifuge tube and again shaken with 0·4 g. permutite. After a third shaking with permutite the liquid is centrifuged to remove any fine permutite particles and 5 ml. taken for colorimetric determination.

We have carried out the colorimetric determination by means of the Sakaguchi reaction in a manner very similar to that described by Bodansky [1936]. We used neutral grey as a permanent standard, but, as we have found a linear relation between the amount of glycocyamine present and the neutral grey reading for amounts of glycocyamine between 0·02 and 0·10 mg., and as the colour obtained is stable up to 30 min. or more, simple colorimetry with a glycocyamine standard should be as accurate as our method. We find that:

$$\text{Glycocyamine (mg.)} = 0{\cdot}00888\ R_{10},$$

where $R_{10}$ is the length in mm. of the column of neutral grey which balances a 10 mm. column of the unknown solution, comparison being made through filter No. 5 on the Leitz colorimeter (509 m$\mu$). This relation holds, however, only when the following procedure is adhered to: 1 ml. 0·04 % $\alpha$-naphthol and 1 ml. 10 % NaOH are added to 5 ml. test solution and the mixture placed in ice for 15 min.; then 0·5 ml. sodium hypobromite solution is added with stirring, followed exactly 15 sec. later by 1 ml. ice-cold 40 % urea solution. 10 ml. ice-cold distilled water are then added as rapidly as possible, and the colorimetric comparison is made.

We have found it convenient to use an apparatus consisting of two pipettes of appropriate size connected by means of T-tubes and taps to a syringe for filling and to a pressure bottle for expulsion of the solutions. A separate lead from the pressure bottle to a glass tube drawn out to a fine tip provides a means of stirring the solutions. It is also advisable to have a jacket round the urea pipette which can be filled with ice-water. With this apparatus we obtained the relation between colour development (in terms of $R_{10}$: see above) and the time interval between the addition of hypobromite and urea which is illustrated in Fig. 1. It is clear that the maximum reproducibility is to be expected when the flatter portions of the curves are used, and we have therefore chosen 15 sec. interval.

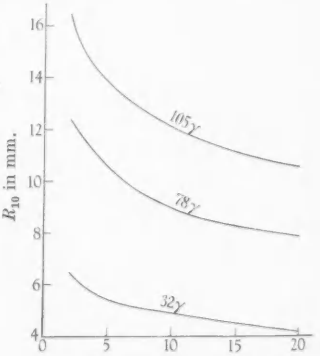


Fig. 1.

Despite the apparent complexity of the procedure, Table I shows that very considerable regularity in the recovery of glycocyamine submitted to the procedure can be obtained. The probable error of individual determinations is only $\pm 2{\cdot}1$ % of the glycocyamine added, or $\pm 2{\cdot}3$ % of the glycocyamine recovered. However, as we have adopted the practice of using quadruplicate determinations for all analyses, the figures in the last column of Table I are the most important. They show that the recovery calculated from the mean of quadruplicate analyses is $89{\cdot}9 \pm 0{\cdot}6$ %. Taking into account the size of the series from which these statistics are calculated, there is less than one chance in fifty of the observed recovery differing by more than $\pm 2{\cdot}4$ % from 89·9 %.

Observations on the amount of glycocyamine left in a solution after treatment with fuller's earth and on the amount of glycocyamine found in the water used for washing show that approximately 10% of the glycocyamine used can be found in these situations, so that it appears that 90% constitutes the maximum practicable recovery.

Table II illustrates the specificity of the method. The figures in the column "glycocyamine found" are obtained by dividing the mean amount recovered by 0·899 (i.e. by the mean recovery). It will be seen that, so far as the two most probable interfering substances are concerned, the method is very highly specific. We have not had the opportunity to test possible interfering substances other than arginine and creatine, but it seems highly probable that those substances which Bodansky [1936] found to be without effect on the original Weber technique will be without effect on our method. These substances were: sarcosine, choline, betaine, creatinine, glycine, alanine, histidine, guanidine and methylguanidine.

Table I. *Recovery of glycocyamine by method A*

| | | % recovery | |
|---|---|---|---|
| Glycocyamine added (mg.) | Glycocyamine recovered (mg.) | Individual determinations | Mean of quadruplicates |
| 0·101 | 0·090 | 89·2 | |
| | 0·090 | 89·2 | |
| | 0·091 | 90·2 | |
| | 0·091 | 90·2 | 89·7 |
| 0·157 | 0·137 | 87·3 | |
| | 0·145 | 92·4 | |
| | 0·140 | 89·2 | |
| | 0·147 | 93·4 | 90·6 |
| 0·202 | 0·182 | 90·1 | |
| | 0·182 | 90·1 | |
| | 0·182 | 90·1 | |
| | 0·184 | 91·1 | 90·3 |
| 0·270 | 0·245 | 90·7 | |
| | 0·238 | 88·2 | — |
| 0·321 | 0·284 | 88·5 | |
| | 0·286 | 89·1 | |
| | 0·287 | 89·4 | |
| | 0·288 | 89·7 | 89·2 |
| 0·404 | 0·362 | 89·6 | |
| | 0·358 | 88·6 | |
| | 0·358 | 88·6 | |
| | 0·369 | 91·3 | 89·5 |
| | Means | 89·8 ± 2·1 | 89·9 ± 0·6 |

Table II. *Specificity of method A*

| Glycocyamine added (mg.) | Arginine added (mg.) | Creatine added (mg.) | Glycocyamine found (mg.) |
|---|---|---|---|
| 0·281 | 0·100 | — | 0·286 |
| 0·221 | 0·415 | — | 0·222 |
| — | 0·415 | — | 0·000 |
| 0·206 | 0·415 | 0·848 | 0·206 |
| — | 0·415 | 0·848 | 0·000 |

*Method B*

In tissue extracts, in which the final salt concentration is not more than 0·4%, small amounts of arginine and creatine can be removed by treatment with permutite, and it is therefore possible to dispense with the adsorption on

fuller's earth. The shortened procedure is as follows: The metaphosphoric acid used as a protein precipitant is removed with baryta, the excess baryta is removed with $H_2SO_4$, and an aliquot of the supernatant fluid is treated with basic lead carbonate and permutite, all the steps being carried out as in method A.

The quantitative character and specificity of this method are illustrated in Tables III and IV respectively.

Table III. *Recovery of glycocyamine by method B*

| Glycocyamine (mg.) | | |
|---|---|---|
| Added | Recovered | % recovered |
| 0·101 | 0·100 | |
| | 0·101 | |
| | 0·101 | |
| | 0·101 | 99·75 |
| 0·126 | 0·124 | |
| | 0·127 | |
| | 0·127 | |
| | 0·128 | 100·05 |
| 0·252 | 0·253 | |
| | 0·252 | |
| | 0·251 | |
| | 0·253 | 100·10 |
| 0·375 | 0·367 | |
| | 0·376 | |
| | 0·378 | |
| | 0·378 | 99·95 |
| | Mean | 99·97 ± 0·15 |

Table IV. *Specificity of method B*

| Glycocyamine added (mg.) | Arginine added (mg.) | Creatine added (mg.) | Glycocyamine recovered (mg.) |
|---|---|---|---|
| 0·250 mg. in Ringer-Locke (salt conc. about 1%) | 0·015 | — | 0·251 |
| | 0·030 | — | 0·263 |
| | 0·050 | — | 0·272 |
| | 0·070 | — | 0·273 |
| | 0·087 | — | 0·270 |
| 0·155 mg. in muscle extract (salt conc. about 0·4%) | 0·024 | — | 0·156 |
| | — | 0·510 | 0·155 |
| | — | — | 0·155 |

As we were able to show that, in the absence of added glycocyamine, perfusates and heart extracts prepared in the manner described by Davenport *et al.* [1938] gave no colour when submitted to method B, we have used this method in the investigation of the relation between glycocyamine disappearance and creatine appearance during perfusion of isolated hearts which is described in the paper referred to above.

## B. IMPROVED DETERMINATION OF ARGININE

By R. B. Fisher and A. E. Wilhelmi

The procedure of Jorpes & Thorén [1932] for the colorimetric estimation of arginine which we adopted earlier [Fisher & Wilhelmi, 1937] has been modified in the light of facts elicited in the development of the method for glycocyamine

[Part I above] to give (*a*) more accurately reproducible, and (*b*) more stable colours. The changes in procedure consist first, in adding the urea *exactly* 15 sec. after the addition of hypobromite, by the method described above, and second, in adding 10 ml. ice-cold distilled water immediately after the addition of the urea, as recommended by Weber [1935] for glycocyamine. Further, we have found that the samples need be kept in ice for only 15 min. after the addition of alkali and α-naphthol before the colour is developed. After this interval no further change with time (up to 2 hr.) seems to occur, so that long series of consecutive estimations may be run quite safely.

Colorimetry is done with neutral grey solution as a standard in the manner described by Fisher & Wilhelmi [1937]. The relationship between amount of arginine present and depth of neutral grey for a range extending from 25 to 110$\gamma$ is

$$Y = 5{\cdot}60\,x^{1{\cdot}06},$$

where $Y$ is the amount of arginine in $\gamma$, and $x$ is the matching depth of neutral grey in mm. when the unknown is set at 10 mm. The standard error of an individual estimation is $\pm 2{\cdot}3\,\%$, showing a marked improvement over the former method.

Table V. *Change in apparent arginine with increasing time after development of colour*

| Arginine present ($\gamma$) | Arginine found ($\gamma$) | | | | Total change ($\gamma$) |
|---|---|---|---|---|---|
| | Min. 3 | 25 | 45 | 60 | |
| 27·1 | 27·6 | 30·8 | 30·3 | 33·8 | +6·2 |
| 27·1 | 28·6 | 28·6 | 29·0 | 29·2 | +0·6 |
| 81·4 | 84·4 | 81·9 | 78·4 | 77·5 | −6·9 |
| 81·4 | 82·9 | 79·4 | 78·9 | 77·0 | −5·9 |

The stability of the colour is illustrated in Table V. It is apparent that the colours are stable enough to allow time for the preparation of standards in instances where some method of absolute colorimetry is not at hand. Although the arginine-colour relationship is not strictly linear, over short ranges departure from linearity is not serious, and no very great error is introduced in using standards of nearly the same strength as the unknown. Since the colours are stable enough to allow time for the preparation and development of an appropriate standard, the estimations can, if necessary, be carried out with reasonable accuracy by simple colorimetry.


## Summary

Improvements in Weber's method for the determination of glycocyamine are described which result in increased sensitivity and reproducibility. The probable error of the mean of quadruplicate analyses is $\pm 0{\cdot}6\,\%$.

A shorter and less specific method is described for use in certain restricted circumstances. The probable error of the mean of quadruplicate analyses is $\pm 0{\cdot}15\,\%$.

The method of Jorpes & Thorén for determination of arginine has been modified to give increased stability of colour and an accuracy of $\pm 2{\cdot}3\,\%$.


We wish to express our gratitude to Prof. Peters for his interest in this investigation and to the Christopher Welch Trust for a grant for the purchase of apparatus. One of us (A. E. W.) is indebted to Mrs Katherine Burrell for a maintenance grant.

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# LXXX. A SHAKER FOR QUANTITATIVE ADSORPTION EXPERIMENTS

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(*Received 1 February 1938*)

The essential details of a simple effective shaker for quantitative adsorptions from aqueous solutions, which can be built almost entirely of Meccano parts, are illustrated in the accompanying diagram. A double crank (*D*), driven by a sprocket and chain with appropriate reduction from an electric motor, is coupled to a chain (*C*) which passes round a sprocket (*S*) fixed to a shaft bearing gears (*G*) supported above and below. These gears interlock with other paired gears ($G_1$, $G_2$) in train. When the crank revolves, the chain turns the sprocket first one

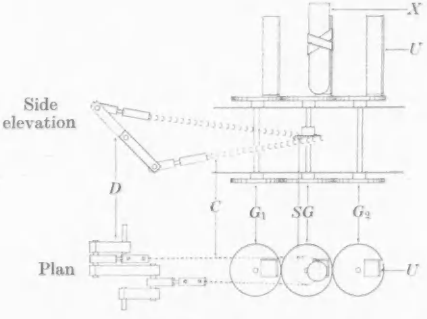


Fig. 1. Semi-diagrammatic representation of shaking mechanism.

way, then the other, and the train of gears is thus set in rapid alternating motion through an arc of a circle. Each upper gear serves as a table to support the adsorption vessel. An upright (*U*) is fixed near the periphery of the gear, and a 25 or 15 ml. centrifuge tube (*X*) is held firmly to it by a broad rubber band.

A machine embodying this principle was devised and used extensively in the development of methods for the estimation of creatinine and glycocyamine [Fisher & Wilhelmi, 1937; Davenport & Fisher, 1938], and it has given satisfactory service, with minimum attention, for over 2 years. It has the advantages that unstoppered vessels can be shaken without loss of their contents and that a large number of vessels can be shaken simultaneously for any length of time. With a shaking radius of about an inch and proper regulation of the speed of shaking, a heavy adsorbent such as fuller's earth can be kept effectively in

suspension in a maximum of 7 ml. of solution in a 15 ml. centrifuge tube, or of 15 ml. of solution in a 25 ml. centrifuge tube, without stoppering the tubes and without loss of their contents by splashing. The ease of operation of the shaker is illustrated by the fact that our machine, with 16 pairs of gears in train, and powered by a 1/30th horse-power electric motor, runs smoothly and efficiently at a maximum crank speed of over 180 r.p.m.

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# LXXXI. THE INFLUENCE OF THIOL-GROUPS IN THE ACTIVITY OF DEHYDROGENASES

By FREDERICK GOWLAND HOPKINS and EDWARD JAMES MORGAN

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(*Received 31 January 1938*)

The work of Heffter [1907] in which the existence of labile thiol groups in tissues was demonstrated, and his enlightened prediction that they would be found to play a noteworthy part in tissue respiration, suffered neglect for several years. In the paper containing the first account of glutathione [Hopkins, 1921] it was pointed out that the presence of that substance in the oxidized no less than in the reduced form accelerated the reduction of methylene blue by partly washed tissues, and that this was because by its reduction and subsequent oxidation it acted as an intermediate transporter of hydrogen from tissue to dye. This seems to have given the first hint that intermediate H transport might be a process proper to living tissues. In this paper it was also shown that a tissue washed completely free from soluble H donators still reduced methylene blue in the presence of GSSG, though no attempt was made to decide on what is then the source of the H. In a later paper however [Hopkins & Dixon, 1922] the significance in this connexion of Heffter's thiol groups in proteins was made clear. It was shown that they are primarily responsible, in a thoroughly washed or heat-denatured tissue, for the reduction of GSSG; being themselves oxidized in the process. Later [Hopkins, 1925], in the course of a study of the influence of glutathione on the oxidation of fats and proteins, it was shown that GSH, in sufficiently concentrated solutions, actively re-reduced the oxidized protein groups, and further that these reversible relations resulted in the establishment of equilibria depending on the relative concentrations of GSH and GSSG in solution and the number and activity of similar groups in the tissue. These relations correspond in effect to an equilibrium reaction between heterologous thiols and disulphides.

$$RSSR + 2R'SH \rightleftharpoons R'SSR' + RSH.$$

Recently Bersin and Steudel [quoted Bersin, 1936] have studied such a reaction in the case of cystine and thiolacetic acid.

Grassmann *et al.* [1929], having the above-mentioned facts in mind, showed that papain and kathepsins can be activated by cysteine or by reduced glutathione; since then many researches have dealt with the influence of thiol compounds on the activity of hydrolysing enzymes. It has become clear, in many cases at least, that thiols can activate such enzymes, and this because of their reducing powers. It is true that the activation observed *in vitro* may in certain cases be due to the circumstance that during its preliminary treatment the enzyme preparation has been partly inactivated by oxidation, and full activity is then restored by contact with (say) GSH. The same enzyme, however, may on occasions be separated without loss of its full activity, and glutathione will then seem to have no functional relations with it. Yet, amid the diverse oxidations

proceeding within the living cell, the properties of glutathione may serve to preserve the activities of any such enzyme, and play such a part in the general equilibrium of the cell. Finally, evidence has accumulated to suggest, or perhaps to prove, that in many cases it is the fixed —SH groups in the protein element of each enzyme which suffer these reversible processes of oxidation and reduction. "The active enzyme is thus to be looked upon as a thiol compound, while its inactive form is a disulphide compound" [Bersin, 1936].

It has indeed been claimed that the activating powers of glutathione, in some cases at least, are due to its ability to form inactive associations with the ions of heavy metals of which the presence inhibits the activity of the enzyme. This view, we think, has been sometimes advanced without adequate evidence. In any case such inhibition may itself be due to the tendency of metals to block, by replacing the H, the —SH groups which are necessary for activity. A sufficient concentration of glutathione (GSH) will restore such thiol groups. If in certain cases GSH thus acts by preventing inhibition by metals, we think such cases will prove to be less common than those in which its reducing powers are concerned. Recent work has extended the field in which the phenomena under review are to be observed. In addition to the activity of proteinases those of lipases, phosphatases, a cerebrosidase and glucosidases have been shown to be related in one way or another to sulphur groupings. Urease and arginase are rather special cases, while the effect of glutathione on glyoxylase activity is, of course, known to depend on relations with the substrate rather than with the enzyme.

As this paper is concerned, not with hydrolytic enzymes, but with dehydrogenases, it does not seem necessary or justifiable to refer in detail to the now extensive literature dealing with the former which has been briefly reviewed above. Bersin, whose own work has contributed much to the subject, has more than once dealt fully and critically with the literature, and in his reviews full references are to be found [Bersin, 1935; 1936].

In the case of the dehydrogenases very little work seems to have been done in this particular field. Wagner-Jauregg & Möller [1935] studied, it is true, the activation of hexosephosphatase and alcoholdehydrogenase, and came to the conclusion that glutathione activates these enzymes by immobilizing inhibitory metallic ions.

A preliminary study, now to be described, of the behaviour of dehydrogenases has shown that, in one at least, it is exceptionally easy to demonstrate the apparent importance of the protein —SH groups for the enzymic activity, and, no less, the influence of glutathione in maintaining the integrity of these groups.

This is the succinic dehydrogenase, with which, and with the glycerophosphate dehydrogenase this paper is chiefly concerned. If an active preparation of this enzyme is first suspended anaerobically at 37° in a solution of GSSG buffered at *p*H 7·4, and if then after a suitable interval it is washed free from the glutathione, the SH groups are found to be oxidized and with their disappearance the activity of the preparation is found also to have completely disappeared. If then the inactivated preparation is treated as before, but now with a solution of GSH replacing the GSSG solution, the protein SH groups are restored and so is the whole original activity of the enzyme. This clear-cut response to the treatment is not displayed by all the enzymes so far studied; but the differences among them seem to be in themselves of no small interest.

## Experimental

The experiments described in this paper were mostly made with the methylene blue technique of Thunberg. As they were chiefly concerned with the preliminary activation of substrate H, and not with H transport, the method is fully adequate. A few determinations of $O_2$ uptake, however, were also made.

When animal tissues were employed as a source of the enzymes they were, as is customary, first thoroughly washed with distilled water till free from H donators. In the case of muscle at least, it proved convenient and adequate to submit the tissue to no further disintegration than that obtained by the use of the Latapie mincer. It can then be filtered off and washed on a Büchner funnel at those stages in the subsequent treatment when previously added glutathione has to be removed (*infra*). If such tissues are more finely ground the washing must be by the centrifuge, which takes longer. With liver and kidney washing by the centrifuge usually proved necessary. When more soluble enzymes are under study (as those from yeast) the glutathione in either form must be added to their solutions and subsequently removed by dialysis. With these however the present paper is but little concerned.

Further details will receive mention in the description of individual experiments.

### *Succinic dehydrogenase*

*Preparation 1.* In general the details described here apply to other experiments and need not afterwards be repeated. The thigh muscles of a rabbit were put through a Latapie mincer, and the mince washed thrice with successive quantities of distilled water, being squeezed out in linen at each stage. Of the washed tissue 3 g. were placed in a Thunberg tube, 50 ml. in capacity, with 10 ml. *M*/10 GSSG, 2 ml. phosphate buffer *p*H 7·4, together with a few drops of $CHCl_3$. The tube was evacuated and kept in the water bath at 37° for 3 hr. The tissue was then filtered off through linen on a Büchner funnel, and washed until completely free from glutathione, now in part reduced. It was then returned to the tube, which now contained 10 ml. *M*/20 GSSG with buffer, and, after evacuation, replaced in the bath for another 3 hr. The tissue was again washed free from glutathione, and, when sucked as dry as possible by the filter pump, it was removed and weighed. It was divided into 2 equal parts, one-half being reserved (*oxidized product*). The other half was replaced in the vacuum tube with buffer and 3 ml. *M*/10 GSH, placed in the bath for 3 hr. and then finally filtered, washed and well drained at the pump (*reduced product*). As equilibria are concerned it is necessary for satisfactory oxidation of the SH groups that the tissue should be treated more than once with the GSSG solution. It is to be noted that the oxidation of the protein SH is a slower process than the reduction of the protein SS. In this experiment the oxidized preparation showed a very faint nitroprusside reaction. In the reduced sample the reaction was as intense as in the original washed tissue.

The tissue used as a control in the determination of the reduction times was not the original washed tissue. In case the enzyme should suffer any spontaneous change of activity when alone, a portion of the original preparation was taken through all the stages of treatment side by side with the other samples but without added glutathione. This was the "original" in the methylene blue experiments. The same plan was adopted in all other experiments.

The reductions were carried out in smaller Thunberg tubes. As each preparation (original, oxidized and reduced) had its own control without donator,

6 tubes were employed. Each tube contained tissue to correspond with 0·5 g. washed muscle, together with 2 ml. phosphate buffer *p*H 7·4, 0·5 ml. *M*/2 succinate and 0·25 ml. water or (in the controls) 0·75 ml. water. To each was added 0·25 ml. *M*/1000 methylene blue. The tubes were evacuated as usual and placed in a bath at 37°. The observed reduction times are given in detail in Table I. The significant figures in this experiment were (with succinate) 11 min. for the original, > 5 hr. for the oxidized tissue, and 9 min. for the tissue when re-reduced.

*Note.* A small technical difficulty easily overcome may be mentioned. The three preparations, owing to the difference in their histories, may acquire slight differences in physical conditions, with the result that the imbibed water content when removed from the filter varies somewhat. The preparations were, however, so handled throughout as to avoid loss of the tissue. As in each case the original weight employed was known, the final weights before use measured the varying degree of moisture present, so that (in this and similar experiments) amounts equivalent to 0·5 g. of the original washed preparation could be employed in each tube. When disintegration is carried no further than by use of the Latapie mincer, the preparation after the exposure to GSSG when tested directly gives no trace of a nitroprusside reaction. Occasionally, though not usually, and only in the case of skeletal muscle, if the oxidized tissue be more finely ground before applying the test, a faint reaction may be obtained. This doubtless is due to a permeability factor. It does not happen with heart muscle. Presence or absence of this slight residuum of SH groups made no appreciable difference in the behaviour of a preparation in the reduction tests. It is likely that the concentration of the enzyme effective in these tests is on the more superficial parts of the relatively coarse particles. When, as in preparations 7 and 9 the original washed tissue was finely ground before being submitted to the influence of GSSG, the nitroprusside reaction was always found to be absent. In the tissue after re-reduction by GSH the reaction was always of an intensity comparable with that of the original washed tissue.

*Preps. 2, 3 and 4.* The details of the procedure used in making these, in each of which the muscles of different rabbits were used, were precisely the same as those of prep. 1, but a larger quantity (with correspondingly larger amounts of GSSG and GSH) was oxidized and reduced in each case, in order to test other enzymes on the same materials (for their behaviour see Table I).

*Prep. 5.* This was made from the liver of the animal of which the muscles yielded prep. 2. In general the treatment was the same as that used in the preparations from muscle. It was found best however to free the minced liver tissue from added glutathione by thorough washing at the centrifuge. It was so washed until the washings gave no more than a trace of reaction with nitroprusside, even in the presence of cyanide.

*Prep. 6.* This was made from a pig's heart obtained soon after death. The tissue was minced with the Latapie and in general received treatment similar to that applied to the skeletal muscle of the rabbit, as described for prep. 1.

*Prep. 7.* This was also from pig's heart. After washing and mincing however it received further treatment. This was exactly that described by Ogston & Green [1935] for the preparation of succinic dehydrogenase, and since used by Dr Green in other experiments. We owe to his kindness and to that of Dr Dewan a supply of preparations so made. In the table the preparations thus disintegrated are distinguished by a star.

*Prep. 8.* From the kidney of a pig brought while still warm from the slaughter-house. The general treatment of the tissue was again similar to that used for preps. 1–6, but the centrifuge was used for washing at each stage.

*Prep. 9.* From rabbit muscle. The tissue was used for experiment after receiving the treatment referred to under the head of prep. 7, and in the experiments the same technique was employed. In this case when, at the necessary stages, the tissue was being washed by the centrifuge to remove glutathione, the

material refused to settle satisfactorily from its suspension in the phosphate-buffered fluid. The addition of 5 or 6 drops of 10% $CaCl_2$ however induced separation.

All the results obtained for the succinic acid dehydrogenase are assembled in Table I.

Table I. *Succinic dehydrogenase*

| Prep. no. | Animal | Tissue | Reduction times: Original min. | Oxidized hr. | Re-reduced min. |
|---|---|---|---|---|---|
| 1 | Rabbit | Muscle | 11 | 3 + + | 9½ |
| 2 | ,, | ,, | 18 | ,, | 21 |
| 3 | ,, | ,, | 9 | ,, | 9 |
| 4 | ,, | ,, | 11 | ,, | 10 |
| 5 | ,, | Liver | 16 | ,, | 11 |
| 6 | Pig | Heart | 5 | ,, | 5½ |
| 7* | ,, | ,, | 10½ | ,, | 12 |
| 8 | ,, | Kidney | 34 | ,, | 13 |
| 9* | Rabbit | Muscle | 20½ | ,, | 20½ |

It will be seen from the table that in every case so far studied, when succinate was the substrate, exposure to the influence of GSSG removed all dehydrogenase activity from the tissue. On the other hand, subsequent exposure to GSH re-established the activity in all cases, and, in the majority, to the same degree of activity as that displayed by the original tissue. In the case of the inactivated product it will be noticed that for convenience the reduction time is given in all cases as 3 hr. + +. It is difficult in the course of a working day to observe the tubes for longer than this, while to leave them in the bath overnight is undesirable. In every case of an oxidized preparation at the end of 3 hr. the dye remained apparently unaffected, the colour being still that of the control without added donator.

To the evidence yielded by the methylene blue technique may be added that obtained when a Keilin system is established with the tissue preparation, and the $O_2$ uptake observed in each case. In the first place, it proved easy to show that a tissue preparation oxidized by GSSG entirely fails in anaerobic conditions to reduce cytochrome, whereas after reduction by GSH the same tissue reduces it equally with the original preparation. It was not surprising therefore to find, when oxidized and reduced preparations were placed side by side in Barcroft manometers, each containing a buffered solution of succinate and a supply of cytochrome, that the latter showed a steady uptake of $O_2$ and the former no uptake at all. The necessary indophenol oxidase was of course contained in the tissue itself. This seems to undergo some loss of activity during the treatment of the preparation, and the $O_2$ uptake in the case of the re-reduced tissue was slower than in that by the original, but nevertheless quite active and steady.

*The α-glycerophosphate dehydrogenase*

Most of the preparations used in the study of the succinic system were used at the same time for testing the effect of the treatment they had received on the activity of the glycerophosphate enzyme. Thunberg tubes, otherwise quite identical in their contents except for the nature of the substrates (succinic acid and the glycerophosphate respectively), were placed in the water bath simultaneously, or observations on both were made with but a short interval between them. With this complete similarity in conditions it was striking to observe the difference in the two cases. Instead of the disappearance and reappearance of

activity so invariable in the succinate system, in the case of the glycerophosphate system oxidation and reduction produced no effect at all. As will be seen in Table II, the reduction times of the original, the oxidized and the re-reduced preparations were the same. The agreement indeed was striking and of course quite unexpected in advance. (The numbers attached to the preparations in Table II refer to the same preparations as do the similar numbers in Table I.)

Table II. *α-Glycerophosphate dehydrogenase*

| Prep. no. | Animal | Tissue | Reduction times (min.) Original | Oxidized | Re-reduced |
|---|---|---|---|---|---|
| 2 | Rabbit | Muscle | 5 | 6 | 6 |
| 3 | ,, | ,, | 8 | 7½ | 8 |
| 9* | ,, | ,, | 7 | 7 | 7 |
| 6 | Pig | Heart | 32 | 32 | 32 |
| 8 | ,, | Kidney | 20 | 22 | 20 |

The agreement in the results yielded by each enzyme individually, and the sharp difference shown when the collective results are compared, add to confidence in the technique employed. The results seem also to establish as a fact the existence of a real difference in the configuration of two enzymes which otherwise have properties so similar that it was at one time thought they might even be identical. Their specificity in relation with their substrates would seem, however, to be based on some fundamental differences in their constitution, or in the mechanism of their action. The presence of a thiol group is apparently necessary for the activity of the one while having no apparent function in the case of the other. This point receives some further notice later. It may be added that the results got by the methylene blue technique with the glycerophosphate enzyme were, as with the succinate, confirmed by constituting cytochrome-indophenol-systems with the oxidized and reduced forms. In the case of the former enzyme both showed a notable $O_2$ uptake.

*Other enzymes*

No such clear-cut results were obtained in preliminary studies of two other enzymes which function in the activation of substrate hydrogen. These were the lactic acid and the hexosediphosphate enzymes which as dehydrogenases require of course the presence of a coenzyme for their activity. When the various preparations of muscle tissue used in the experiments already described were employed in the same way as before but with lactic acid as the hydrogen donator the results, though irregular, suggested that the enzyme behaved similarly to the succinic dehydrogenase, but was more resistant to the influence of glutathione. The reduction time of the tissue, after exposure to GSSG for periods similar to those otherwise employed, was slower than in the case of the original tissue, but it still reduced methylene blue; while after treatment with GSH reduction was quickened, but the time taken did not fall to that of the original, occasionally the effects were small. When hexosediphosphate was the donator similar behaviour was observed. Further work is necessary before definite statements can be made concerning these two enzymes, but the results again illustrate the existence of fundamental differences in the specific make-up of different dehydrogenases.

It became clearly desirable to test the behaviour of yeast dehydrogenases when treated on similar lines. In the case of soluble enzymes however, the technical difficulties proved greater than when washed tissues were employed. The glutathione in both forms had of course to be added to the enzyme solutions,

and its subsequent removal by dialysis proved at each stage to be a slow process. The whole treatment occupied therefore a long time during which the activity of the enzymes became much reduced. In further experiments an endeavour will be made to employ a different technique for the removal of glutathione, possibly with the use of ultrafiltration.

*Oxidation and reduction by cystine and cysteine*

The question might arise as to whether the influence of glutathione on an enzyme such as the succinoxidase depends on some relation between the structure of the tripeptide as a whole and the structure of the enzyme, or whether the S groups alone are directly concerned. It seemed therefore desirable to ascertain if cystine and cysteine could act respectively as oxidant and reductant. The slight solubility of the former was expected to involve difficulties, but success was easily reached in the following way. Of pure cystine 0·12 g. was dissolved by boiling it in 30 ml. phosphate buffer at *p*H 7·6. It was then placed in the water bath until it had cooled to 37°, no separation occurring. It was then poured straightway into a large Thunberg tube already in the water bath containing 6 g. minced and washed pig's heart with a few drops of $CHCl_3$. The tube was then evacuated and left in the bath for 4 hr. No visible separation of cystine occurred; it remained apparently in supersaturated solution. A portion of this product after washing at the pump was then reduced on the lines of previous experiments using *M*/10 cysteine as the reductant. Tested for their reduction times the preparations gave the following data: original $4\frac{1}{2}$ min., oxidized 3 hr. + +, reduced 5 min. The cystine and cysteine adequately replaced glutathione.

*Reaction with iodoacetate*

Since the observations of Quastel & Wheatley [1932] and Quastel [1933] on the glyoxalase system and the very complete study published almost simultaneously by Dickens [1933; 1, 2], it has been generally believed that when the activities of tissues *in vitro* prove to be inhibited by iodoacetic acid, it is because the acid interacts with SH groups, be they in the substrate or the enzyme.

In a study of the action of the halogen-acid on various dehydrogenases Dixon [1937] found that in the conditions of his experiments the succinic dehydrogenase was relatively insensitive to its action. Incubation of a preparation from muscle with a *M*/100 solution for 10 min. reduced its activity by 11 % only. If therefore the acid interacts with no groups in proteins other than thiol groups, it might seem that Dixon's results are not to be easily reconciled with ours. This, we think, does not follow.

A tissue preparation as generally employed contains, of course, in addition to the specific protein of the enzyme studied, a large proportion of other protein material carrying thiol groups. If such a preparation be treated exactly on the lines described by Dixon, it will be found afterwards to give still a well-marked nitroprusside reaction.

Rapkine [1933] has shown that the reaction of the acid with the SH groups of proteins when in the physical condition of those in a washed tissue preparation is a slower process than the action with soluble thiol compounds, and it is clear that if it is to react with every thiol group present during any given incubation time, there must be some definite relation between an effective concentration of the acid and the total amount of thiol proteins present. Smythe [1936] has shown, it is true, that in the case of urease (using iodoacetamide instead of the acid) for the complete inactivation of the enzyme it is apparently not necessary

to destroy more than, at most, half of the SH groups present. We do not, of course, yet know whether these groups in different enzymes, or in other proteins, do or do not vary in their rate of reaction with iodoacetic acid.

In any case it is clear from the following experiments that our results do not really conflict with those of Dixon.

A preparation from pig's heart had been minced and washed as in the other experiments. A portion was incubated with $M/100$ iodoacetic acid for 10 min. as in the experiments of Dixon [1937], and a second portion was similarly incubated for 30 min. The reduction times with methylene blue and succinate (0·5 g. tissue in each case) were then determined, using the same technique as that employed by Dixon. They were as follows: *untreated*, 4 min.; *incubated 10 min.*, 7 min.; *incubated 30 min.*, 40 min. A preparation which had been oxidised and re-reduced gave the following reaction times : *untreated*, 7 min.; *incubated 10 min.*, 27 min.; *incubated 30 min.*, $>2$ hr. The effect of incubation time is therefore very great.

A second preparation was made from rabbits' heart muscle by the method of Ogston & Green and used as a suspension. It retained an adequate amount of cytochrome for estimations of $O_2$ uptake. When 0·5 ml. was shaken in a Barcroft manometer together with 0·2 ml. $M/10$ succinate, the system took up 215 $\mu$l. $O_2$ in 15 min. When the same amount of enzyme was first incubated with $M/300$ iodoacetic acid for 30 min., its activity judged by the induced $O_2$ uptake was reduced by some 20 % only. When however the same amount of enzyme was incubated with $M/100$ acid for 30 min. and used as before, the $O_2$ uptake was reduced almost to zero. On the other hand, when the amount of enzyme was doubled (1 ml.) and incubated with the $M/100$ solution for 15 min., its activity was still nearly half that of the original. The $O_2$ uptake experiments are perhaps less significant than the reduction experiments though they certainly support them. In the former it might be said that the effect is due or partly due to the effect of iodoacetic acid on the indophenol oxidase.

It seems sure that in Dixon's experiments the ratio of the amount of enzyme preparation to the concentration of iodoacetic acid was probably too high, and the incubation time certainly too short, for the effect on activity to be considerable.

On the other hand, an explanation of the behaviour of the $\alpha$-glycerophosphatase is more difficult to find. Our experiments seem to prove that it is wholly independent of SH groups whereas in Dixon's its activity showed 60 % reduction on incubation with $M/100$ iodoacetic acid. This case seems to present an interesting problem for future study.

## Discussion

The experiments described show unequivocally in the case of the succinic dehydrogenase that when the enzyme is exposed to the oxidizing influence of GSSG its activity disappears simultaneously with the disappearance of the tissue-thiol groups, while both reappear together under the reducing influence of GSH. It might perhaps be suggested that this does not furnish a complete proof that the thiol groups are essential for the activity of the enzyme. Might not the protein-thiol groups act merely as indicators of the occurrence of the oxidation and reduction which is shared by other and actually essential groupings on the enzyme surface? This however is exceedingly unlikely. GSSG is a very mild oxidizing agent. The redox pair, —SS— $\rightleftarrows$ 2SH—, is a relatively negative system. There is indeed evidence to show that as an oxidant for proteins GSSG is specific for the thiol groups. Work by Mirsky & Anson [1935] for instance (though it is true they were studying denatured proteins, and using cystine as the oxidant)

seems to show that the total amount of hydrogen transferred to the oxidant is at any rate an approximate measure of the number of "fixed" thiol groups in a protein. Similar results have been obtained in this laboratory. It seems sure that the integrity of a thiol group (or groups) in its structure is essential for the activity of the succinic dehydrogenase. This assumption receives indirect support at least from the cumulative evidence derived from the study of hydrolysing enzymes.

Equally unequivocal evidence is given by our experiments that the activity of the α-glycerophosphatase is not based on the influence of sulphur groupings, or, at least, that it does not require the integrity of thiol groups. Oxidation of these has not the slightest effect upon the activity. It is remarkable that so sharp a distinction should be found between two dehydrogenases which in most respects—including their independence of a coenzyme—show very similar qualities.

The experiments described in this paper have proved the unmistakable importance of thiol groups only in the case of the succinic enzyme. But this is perhaps the most typical among dehydrogenases; it is widely distributed among animal tissues, and the succinate system seems to hold a prominent position in the chemical economy of living cells. If thiol groups play a leading part in the mechanism of its action their influence will yield an objective illustration of an aspect of specific structure in correlation with specific functions.

It is of course possible that other dehydrogenases may depend for their activity on the integrity of thiol groups. This however, is not yet proved in the case either of the lactic acid or of the hexosediphosphate dehydrogenase. These do not show that complete indifference to the effect of oxidation and reduction which is displayed by the α-glycerophosphate enzyme, but the effects observed in their cases were not clear-cut, and for some reason varied with different preparations. Further studies of these and other dehydrogenases are in progress.


## Summary

The activity of the succinic dehydrogenase is completely abolished when a preparation containing it is placed in a solution of GSSG at *p*H 7·6 and incubated. When, after filtering off and washing, the preparation is next placed in a similar solution of GSH the activity of the enzyme is completely restored. The first treatment involves the removal of the protein SH groups by oxidation and the second their restoration by reduction.

When, on the other hand, similar preparations containing the α-glycerophosphate dehydrogenase are treated in the same way there is no effect whatever on the activity of this enzyme.

These facts and the probable indispensability of the SH group for the activity of the former enzyme are discussed.

The behaviour of certain other dehydrogenases was studied, but requires further elucidation.

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# LXXXII. OBSERVATIONS ON THE SYSTEM ASCORBIC ACID-GLUTATHIONE-ASCORBIC ACID OXIDASE

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Ascorbic acid and glutathione are both highly reducing substances found in living tissues. In the absence of metallic or other catalysts they are not oxidized by atmospheric oxygen. Szent-Györgyi [1928; 1931] described an enzyme which oxidized ascorbic acid but had no effect on glutathione. But if ascorbic acid, glutathione and ascorbic acid oxidase were present simultaneously, oxidation of the glutathione occurred because the enzymically produced dehydroascorbic acid was reduced by the glutathione which was meanwhile itself oxidized.

This interesting interrelation of the three substances was the subject of a later investigation by Hopkins & Morgan [1936]. According to these authors, in the system containing the three components at *p*H 7·4 the ascorbic acid was completely protected from oxidation by the presence of glutathione. Meanwhile the glutathione was oxidized at a rate corresponding to that at which the oxidation of ascorbic acid alone would have proceeded under the influence of the same quantity of enzyme. When the oxidation of the glutathione was complete, and only then, did the oxidation of the ascorbic acid begin. This was only possible if the dehydroascorbic acid were reduced by the glutathione and if this reduction were more rapid than the enzymic oxidation of the ascorbic acid.

In the course of their work Hopkins & Morgan also described the presence of an enzyme in cauliflower juice which catalysed the reduction of dehydroascorbic acid by glutathione. The object of the present study was to investigate this latter reaction. As shown by the results presented below no such enzyme could be found in the cauliflower and cucumber juices used, nor could Hopkins & Morgan's observation regarding the complete protection of ascorbic acid from oxidation by glutathione be confirmed.

## Experimental

### *Materials and methods*

During this investigation the utmost care was taken to avoid contamination by metals of the materials entering the reaction mixtures. The distilled water was twice more redistilled from Jena glass before it was used. Chemicals of the highest available purity were used. As a result the autoxidation of ascorbic acid and of glutathione was negligible at *p*H 6·0. At *p*H 7·4 the autoxidation of the ascorbic acid did not exceed 10 % in 2 hr. At this latter *p*H about 15–20 % of the glutathione was autoxidized in 1 hr. and 35–40 % in 2 hr. This blank was also observed when the glutathione was held at *p*H 7·4 in the buffered solution without ascorbic acid or enzyme. Crystalline natural ascorbic acid and glutathione from F. Hoffmann-LaRoche and Co. were used.

The juices were pressed between hardwood plates in a hand screw-press and did not come into contact with metal parts at all. The cauliflower juice had a similar oxidase activity to that used by Hopkins & Morgan, while the cucumber juice was about twice as active on the volume basis. The different centrifuged samples of cauliflower juice contained about 4 % total solids, the cucumber juice about 3 %. The activity of the juices did not change materially when they were held in the ice-chest for a week or 10 days.

The methods given by Hopkins & Morgan were followed in all details unless noted otherwise. The percentages of total ascorbic acid and glutathione oxidized are shown in the figures rather than the actual titration figures. The values obtained in the control experiments were not subtracted from those presented in the figures because there is some uncertainty whether autoxidation (most likely from traces of metals) would occur to the same extent in the complete reaction mixture. All the experiments were carried out at 30°. Instead of using a shaking machine, the 50 ml. Erlenmeyer flasks containing the reaction mixtures were shaken by hand every 2 min. Preliminary experiments showed that the oxidation of ascorbic acid had the characteristic linear course. The differential titration with Tillmann's reagent and $N/100$ iodine was used for the determination of unchanged ascorbic acid and glutathione.

## Results

### (1) *The ascorbic acid-glutathione-ascorbic acid oxidase system at* pH *6·0*

A study of the system at this *p*H was not reported by Hopkins & Morgan. It was thought important to determine the changes which take place at the reaction most favourable for the enzyme. In the 10 ml. reaction mixture containing 0·5 ml. cucumber juice and 8 mg. ascorbic acid oxidation was complete in 27 min. When glutathione was added to the mixture at the rate of 2 mol. glutathione to 1 mol. ascorbic acid the velocity of the oxidation was hardly influenced at all and even after 2 hr. only 8 % of the glutathione was found oxidized. These results are shown in Fig. 1.

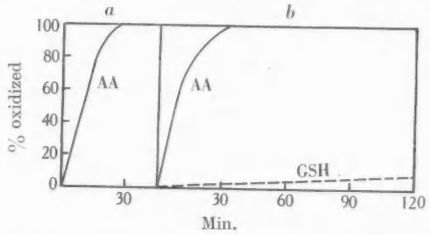


Fig. 1. Enzymic oxidation of ascorbic acid (AA) by cucumber juice at *p*H 6·0. (*a*) Without glutathione (GSH), (*b*) with GSH.

### (2) *The system at* pH *7·4*

In the absence of glutathione the oxidation of the ascorbic acid was complete in 36 min. Addition of glutathione retarded this oxidation and the glutathione was also oxidized. When the glutathione was added in a larger concentration (4 mol. to 1 mol. ascorbic acid) the protecting effect was much more pronounced. The results obtained are shown in Fig. 2. When the experiments were repeated with cauliflower juice the glutathione (2 : 1) gave somewhat greater protection.

In these experiments, the results of which are presented in Fig. 3, the concentration of the enzyme was also varied. In all experiments at *p*H 7·4 both the ascorbic acid and the glutathione were oxidized from the beginning of the reaction. Experiments with dialysed juices gave similar results.

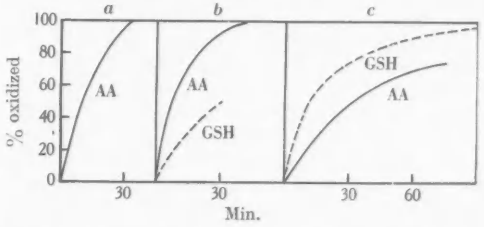


Fig. 2. Enzymic oxidation of ascorbic acid (AA) by cucumber juice at *p*H 7·4. (*a*) AA without glutathione (GSH), (*b*) AA with GSH (2 mol. GSH: 1 mol. AA), (*c*) AA with GSH (4 mol. GSH: 1 mol. AA).

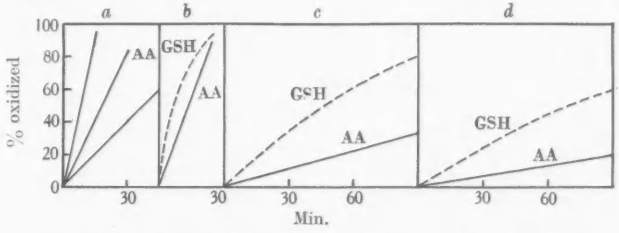


Fig. 3. Enzymic oxidation of ascorbic acid (AA) by cauliflower juice at *p*H 7·4. (*a*) AA with 0·5, 1 and 2 ml. enzyme, (*b*) 2 ml. enzyme and glutathione (GSH) (2 mol. GSH: 1 mol. AA). (*c*) as (*b*) but with 1 ml. enzyme, (*d*) as (*b*) but with 0·5 ml. enzyme.

### (3) *Attempts to catalyse the reduction of dehydroascorbic acid by glutathione with enzymes*

The cause of the failure to repeat the experiments of Hopkins & Morgan may have been the absence from the reaction mixtures of a factor catalysing the reduction of the dehydroascorbic acid by the glutathione. Indeed, experiments with cucumber and cauliflower juices did not indicate the presence of such an enzyme in the material used. It was thought probable that the concentration of the glutathione was not sufficient to produce a rapid reduction. To obtain information on this point ascorbic acid was oxidized by cucumber juice in a manner similar to that described by Hopkins & Morgan in their experiment the results of which are shown in their Fig. 7. Afterwards 1 ml. samples of this reaction mixture were measured into Thunberg tubes containing 0·5 ml. buffer solution at *p*H 6·0. Then in small inner tubes different quantities of glutathione dissolved in 2 ml. water were placed, the Thunberg tubes were evacuated, their contents were mixed and the tubes were kept at 30° for different lengths of time. Afterwards the tubes were opened in such a way that they sucked in 2 ml. *N* HCl to acidify the mixture before air gained access to it. When they were fully opened their contents were diluted with a little water and titrated with Tillmanns's reagent. The results presented in Fig. 4 indicate that the increasing quantities of glutathione did not influence greatly the rate of reduction. It is also apparent that the rate of reduction was slower than the rate of oxidation.

After proving that the failure to obtain faster reduction was not caused by the small quantities of glutathione used, another possibility was considered. The quantity of enzyme solution in the reaction mixtures was only 0·025 ml. in the above experiment and 0·05 ml. when the experiment was repeated with cauliflower juice. In a further set of determinations the 1 ml. samples containing the

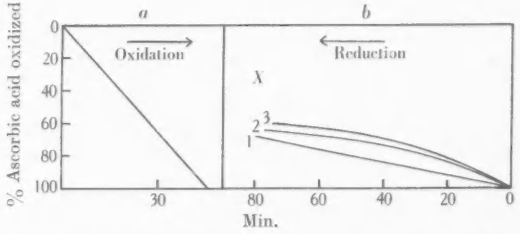


Fig. 4. Regeneration of oxidized ascorbic acid (AA) in presence of glutathione (GSH). (*a*) The course of oxidation at *p*H 6·0, (*b*) the reduction of dehydroascorbic acid (1 mol.) by 10 (curve 1), 20 (curve 2) and 40 (curve 3) mol. GSH at *p*H 6·0. The point marked *X* at 80 min., indicating 70 % recovery of AA, was obtained by the method of Borsook *et al.* [1937], using $H_2S$.

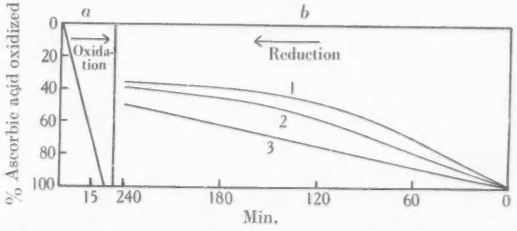


Fig. 5. Effect of additional enzyme on the regeneration of dehydroascorbic acid with glutathione (GSH). (*a*) The course of enzymic oxidation at *p*H 6·0, (*b*) the reduction by GSH (10 mol. GSH: 1 mol. AA) at *p*H 6·0 (curve 1), plus 0·1 ml. cauliflower juice (curve 2) and plus 0·1 ml. cucumber juice (curve 3).

oxidized ascorbic acid were mixed with 1 ml. glutathione solution and buffer, but in addition 0·1 ml. cucumber or cauliflower juice was added to them. These reactions were carried out in Thunberg tubes in the manner previously described. A glance at the curves in Fig. 5 reveals that the added enzyme did not hasten the regeneration by glutathione. On the contrary, the added juice inhibited the reduction slightly.

Experiments also established the fact that dehydroascorbic acid made by oxidation of ascorbic acid with Tillmanns's reagent was reduced by glutathione at a similar rate. Addition of cucumber or cauliflower juice did not influence the velocity of this reaction. The rate of reduction of dehydroascorbic acid by glutathione observed in the above experiments was similar to that described by Borsook *et al.* [1937] for corresponding concentrations at *p*H 6·0.

## Discussion

If glutathione completely protects ascorbic acid from enzymic oxidation and meanwhile glutathione is itself oxidized, the explanation suggests itself that the reduction of dehydroascorbic acid by glutathione is faster than the enzymic

oxidation of ascorbic acid. Hopkins & Morgan proposed this explanation and also presented some experimental data to prove its validity. While no better explanation for the facts observed can be offered by the writer, it should be pointed out that the evidence presented by Hopkins & Morgan was only indirect and partly contradictory. The results obtained with the system ascorbic acid-glutathione-ascorbic acid oxidase, shown by their Fig. 3, were observed at *p*H 7·4. It is doubtful whether the experiment could have been satisfactorily repeated at *p*H 6·0, although Hopkins & Morgan make no statement to this effect. In the experiment presented in Fig. 1 of this paper no protective action of glutathione could be observed. In their experiments the mol. ratio of glutathione to ascorbic acid was 2 : 1 and their results could be obtained with dialysed juice. On the other hand, their experiments to prove that the reduction of the dehydroascorbic acid by glutathione was faster than the oxidation of the ascorbic acid were made at *p*H 6·0 and could not be satisfactorily repeated at *p*H 7·4. Furthermore, the mol. ratios of glutathione to ascorbic acid were 4 : 1 and 8 : 1 and these experiments could not be repeated with dialysed enzyme solutions.

The observation that dialysed cauliflower juice could produce complete protection of ascorbic acid in the presence of glutathione, but could not catalyse the reduction of dehydroascorbic acid by glutathione, makes the explanation given for the mechanism of the reaction somewhat uncertain. The additional observation presented in this paper that the protecting effect of glutathione is (in some cases at least) partial only and not complete makes it appear that some other factor than the enzymic catalysis of the reduction may be involved in the reaction.

It should be stated that the contradictory character of some of the results of Hopkins & Morgan was mentioned in their paper. The purpose of the present discussion is not to point these out but rather to contribute to our knowledge of this interesting but apparently rather complicated interrelation of the three substances. It is the author's desire, furthermore, to call attention to the fact that the observations of Hopkins & Morgan could not all be duplicated under conditions in which the only apparent difference was in the vegetable juices used as sources of ascorbic acid oxidase.

Further work is needed to explain the cause of this discrepancy as well as some of the points not entirely clarified by the work of Hopkins & Morgan.

## Summary

Glutathione added to solutions of ascorbic acid containing ascorbic acid oxidase (cucumber and cauliflower juice) had no effect on the oxidation of the ascorbic acid at *p*H 6·0. At *p*H 7·4 the glutathione inhibited the oxidation of ascorbic acid and both ascorbic acid and glutathione were oxidized simultaneously.

The reduction of dehydroascorbic acid by glutathione was not catalysed by the cucumber and cauliflower juices used in this work.

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# LXXXIII. COENZYME FACTOR—A NEW OXIDATION CATALYST[1]

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Reduced coenzyme I reacts slowly with carriers such as methylene blue, flavin, cytochrome *c*, etc. In presence of flavoprotein these reactions are considerably accelerated. Our previous studies on the lactic, malic and β-hydroxybutyric dehydrogenases of animal tissues [cf. Green & Brosteaux, 1936; Green, 1936; Green *et al.* 1937, 1] have shown that the reaction between reduced coenzyme and carriers was extremely rapid in presence of the dehydrogenase solutions. Euler and his colleagues in a long series of papers [cf. Euler, 1936 for a resumé] have maintained that flavoprotein was the catalyst in dehydrogenase preparations of animal origin and that flavoprotein was in fact an indispensable component of all coenzyme dehydrogenase systems. Their conviction of the essential nature of flavoprotein led them to classify all coenzyme dehydrogenase systems as "Flavinenzym Systemen". Our own experience has been in direct contradiction to this interpretation of the Euler school. We have consistently failed to obtain any evidence either of the presence of any detectable amounts of flavoprotein in any of our preparations or of any significant catalytic action of flavoprotein when added to the complete dehydrogenase systems. In the analysis of the β-hydroxybutyric dehydrogenase of pig heart [cf. Green *et al.* 1937, 1] we were able to show indirectly that the dehydrogenase preparation contained an enzyme which catalysed the oxidation of reduced coenzyme by molecular oxygen. Later the existence of the "coenzyme oxidase" as this enzyme was called, was established beyond any doubt by a direct spectrophotometric method [cf. Green & Dewan, 1937].

The full implications of the "coenzyme oxidase" were not clear to us until we had occasion to compare the properties of mutases and dehydrogenases. The mutases are enzymes which catalyse the oxidation of their substrates by means of α-ketonic acids [cf. Green *et al.* 1937, 2]. Coenzyme I is an essential component of all the known mutase systems. The triosephosphate mutase for example catalyses the oxidation of triosephosphate by oxaloacetate. The mechanism of this oxidoreduction consists in the coenzyme undergoing a cycle of reduction by triosephosphate and oxidation by the ketonic acid. This is in effect a coenzyme-linked reaction between two dehydrogenase systems, viz. the triosephosphate and the malic dehydrogenases. It should therefore be possible to use the triosephosphate mutase as a typical coenzyme dehydrogenase and, by replacing oxaloacetic acid as oxidant with methylene blue or flavin, to obtain the oxidation of reduced coenzyme by these carriers. However, the results of such experiments were invariably negative. It was thus clear that the mutase preparations lacked the agent necessary for catalysing the oxidation of reduced coenzyme. When small amounts of the usual dehydrogenase preparations were added to that of the mutase, vigorous oxidation of triosephosphate via coenzyme and carrier took

[1] A preliminary note has already been published in *Nature* (1937, **140**, 1097).

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place. Clearly our dehydrogenase preparations supplied the missing coenzyme factor. The question naturally arose whether dehydrogenases freed from the factor would be incapable of catalysing the oxidation of reduced coenzyme by carriers. Such was indeed found to be the case.

The oxidation of metabolites in coenzyme-dehydrogenase systems may be regarded as a two-stage process: (1) oxidation of the substrate and reduction of coenzyme, and (2) oxidation of the coenzyme and reduction of the carrier. The dehydrogenase catalyses the oxidation of the substrate; the coenzyme factor catalyses the oxidation of the reduced coenzyme.

The relation between the coenzyme factor and the "coenzyme oxidase" referred to above can now be easily understood. "Coenzyme oxidase" is a complex system containing the following components: (1) coenzyme factor, (2) cytochromes *a* and *b* and (3) cytochrome oxidase. The coenzyme factor catalyses the oxidation of reduced coenzyme by cytochromes *a* and *b*, and the cytochrome oxidase catalyses the oxidation of the reduced cytochromes by molecular oxygen. Thus in the reaction of coenzyme dehydrogenase systems with molecular oxygen three catalysts are involved in the following sequence:

$$\text{substrate} \xrightarrow{\text{dehydrogenase}} \text{coenzyme} \xrightarrow[\text{factor}]{\text{coenzyme}} \text{cytochromes } a \text{ and } b \xrightarrow[\text{oxidase}]{\text{cytochrome}} O_2.$$

The arrows indicate the direction of transfer of hydrogen. Since the last stage is cyanide-sensitive the overall reaction is also cyanide-sensitive.

Recently Adler *et al.* [1937], following on the original observations of Green *et al.* [1937, 1] and Green & Dewan [1937], have presented confirmatory evidence of the existence of the coenzyme factor in animal tissues. Adler *et al.* seem to have made the discovery quite independently; from their preliminary report they appear to have made substantial progress in the study of the coenzyme factor.

### I. *The catalytic oxidation of reduced coenzyme I*

A solution of reduced coenzyme I can be prepared by adding hydrosulphite to a bicarbonate solution of the oxidized coenzyme, incubating for 15 min. at 38° and then aerating vigorously for several hours to convert the residual hydrosulphite or sulphite to sulphate [for details of procedure cf. Green & Brosteaux, 1936; and Green & Dewan, 1937]. The reaction between reduced coenzyme and methylene blue carried out anaerobically in Thunberg tubes is rather slow (Table I). On addition of a phosphate extract of washed and ground

Table I. *The reaction between reduced coenzyme I and hydrogen acceptors*

| | Reduction time in min. |
|---|---|
| Reduced coenzyme (10 mg.) + methylene blue (1 mg.) | 14 |
| Reduced coenzyme + methylene blue + crude muscle extract (2·5 mg.) | 0·5 |
| Reduced coenzyme + methylene blue + boiled muscle extract (2·5 mg.) | 12 |
| Reduced coenzyme + methylene blue + flavoprotein (2 mg.) (2 mg. ≒ 12 $\gamma$ lactoflavin phosphate) | 1·0 |
| Reduced coenzyme (10 mg.) + flavin (1 mg.) | 40 |
| Reduced coenzyme + flavin + muscle extract (2·5 mg.) | 4 |

skeletal or cardiac muscle, the reduction of methylene blue is a matter of a few seconds. The catalytic effect disappears when the extract is previously boiled. Flavoprotein shows the same effect, but there is no means of comparing the relative efficiencies of the two catalysts in absence of data on the molecular

strength of the coenzyme factor. Methylene blue can be replaced with flavin or cytochrome and the catalytic effect of the factor is substantially the same.

## II. *Properties of the factor*

The most convenient sources for preparation of the coenzyme factor are rabbit skeletal muscle and pig heart. The following procedure is employed in the two cases. The fresh muscle tissue (*ca.* 400 g.) is minced in a Latapie and washed exhaustively with tap water. For this purpose it is best to suspend the mince in about 10 l. of water and to stir vigorously for 5 min. The suspension is filtered through muslin and the wash water discarded. The process is repeated six times. The thoroughly washed mince is then mixed with sand and 300 ml. *M*/50 phosphate buffer *p*H 7·2, and ground to a fine paste in a mechanical mortar. The mixture is squeezed through muslin—the pulp being discarded. The filtrate is centrifuged for a minute to sediment fine particles of sand or cellular debris. The solution is then brought to *p*H 4·6 (just yellow to chlorophenol red) by cautious addition of acetic acid. The precipitate is centrifuged and the supernatant fluid is discarded. If the precipitation is carried out correctly the sedimentation is sharp and no difficulty is experienced in pouring off the supernatant. The precipitate is suspended in 500 ml. *M*/50 phosphate buffer and ground in a mortar to break up any lumps. The suspension is then centrifuged at 3000 r.p.m. for 20 min. The supernatant fluid is discarded. This washing procedure is repeated at least three times in order to remove the soluble dehydrogenases which adhere mechanically to the coenzyme factor. The thrice washed sediment is finally suspended in 100 ml. *M*/10 phosphate buffer *p*H 7·2.

As the method of preparation indicates, the coenzyme factor is associated with insoluble particles which can be easily centrifuged out of suspension and thus separated from the soluble dehydrogenases. Successive washing by suspension and centrifuging is necessary because of the tendency of the soluble dehydrogenases to adhere to the insoluble particles. The final suspension contains high coenzyme factor activity and little or no dehydrogenase activity. Unfortunately with prolonged washing there is a fall in factor activity—thus setting a limit to the amount of purification which can be effected by the above procedure.

Comparison of the activities of the suspensions before and after washing shows that about 60 % of the original activity has disappeared in the course of the manipulations. This loss is not due to destruction. Just as is the case for other insoluble enzymes, the particles with which the coenzyme factor is associated show all gradations of size. The gross particles are easily centrifuged whereas some of the smaller particles are so highly peptized that only prolonged centrifuging at 20,000 r.p.m. suffices to sediment them.

The separation of the soluble dehydrogenases from the coenzyme factor is technically much simpler than the converse. Fractional centrifuging or filtration is ineffectual because of the tendency of the coenzyme factor to form highly peptized suspensions. Thus far we have been unable to find any one procedure which is successful in all cases. But in general the following treatments either alone or in combination have been used to separate the coenzyme factor from the soluble dehydrogenases: (1) treating for 15–30 min. at 55°, (2) acetone precipitation and drying and (3) filtration through a pad of charcoal and kieselguhr. The efficiencies of these three methods depend upon the relative proportions of the dehydrogenase and the factor. If the dehydrogenase is in excess, destruction of the dehydrogenase is small compared with that of the factor. But with a relatively small amount of dehydrogenase these treatments are obviously unsuitable. Heating to 52–55° for at least 15 min. invariably destroys

the coenzyme factor. It is inadvisable to centrifuge off the precipitate which forms during the heat treatment as there is a tendency for active dehydrogenase to be carried down as well. Precipitation of the dehydrogenase solution with acetone at 0° does not destroy the factor so much as render it insoluble. The dried acetone power is rubbed up with buffer and the suspension is centrifuged hard. This procedure effects considerable separation. Unfortunately, some of the dehydrogenases are destroyed by acetone precipitation. This treatment is not therefore of general application. Filtration through a pad of charcoal and Kieselguhr (equal proportions) clarifies crude dehydrogenase solutions and removes a great deal of the factor activity. But in some cases the dehydrogenase is correspondingly removed.

### III. *The factor effect with the mutases*

The triosephosphate and triose mutases are prepared by acetone precipitation of the aqueous extracts of minced but unground rabbit skeletal muscle (cf. Green *et al.* [1937, 2] for details). The virtue of these mutases as experimental material lies in the fact that the original extract from which they are prepared contains at most traces of the coenzyme factor.

*Triosephosphate mutase.*

The mutase solution in presence of coenzyme I, hexosediphosphate (i.e. triosephosphate) and methylene blue does not absorb any oxygen. The factor solution in presence of the same components is similarly inactive. But with mutase and factor solution together a vigorous oxidation ensues (cf. Fig. 1).

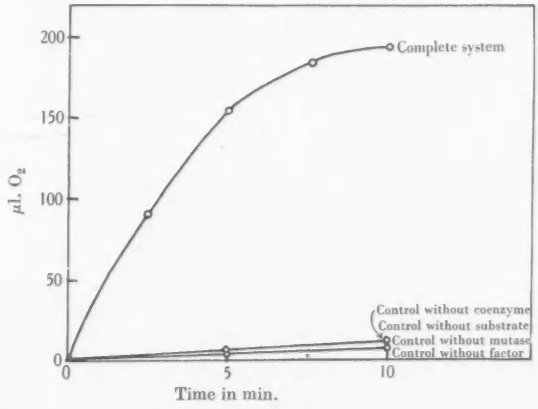


Fig. 1. Factor effect with triosephosphate mutase.

The complete system contained 1·5 ml. mutase (skeletal muscle of rabbit), 0·7 ml. coenzyme factor (pig heart), 0·7 ml. 0·15% coenzyme I, 0·2 ml. 0·5% methylene blue and 0·3 ml. *M*/10 hexosediphosphate. The centre pot of the manometer was filled with KOH paper to absorb $CO_2$.

The mutase solution provides the catalyst for the reduction of the coenzyme; the factor solution provides the catalyst for the oxidation of reduced coenzyme at the expense of the reduction of methylene blue. The significance of the factor effect can be demonstrated in a striking way by carrying out the experiment in Thunberg tubes (cf. Table II).

Table II. *Factor effect with the triosephosphate mutase*

The complete system contained 1 ml. factor, 1 ml. mutase, 1 ml. 0·15% coenzyme I, 0·2 ml. *M*/10 hexosediphosphate and 0·3 ml. 0·5% methylene blue.

| | Methylene blue, reduction time in min. |
|---|---|
| Complete system | 2 |
| Without mutase | ∞ |
| Without factor | ∞ |
| Without substrate | ∞ |
| Without coenzyme | 80 |

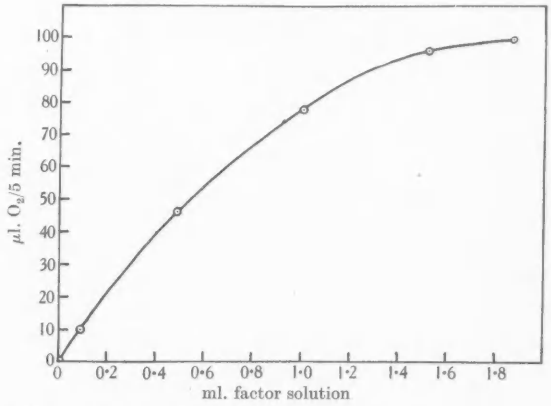


Fig. 2. The effect of varying the amount of coenzyme factor.

The following quantities were used in each manometer: 0·5 ml. mutase, 1 ml. 0·15% coenzyme I, 0·3 ml. 0·5% methylene blue, 0·4 ml. *M*/10 pyrophosphate and 0·3 ml. *M*/10 hexosediphosphate. The centre pots were filled with KOH paper to absorb $CO_2$. Both factor and mutase were prepared from rabbit skeletal muscle.

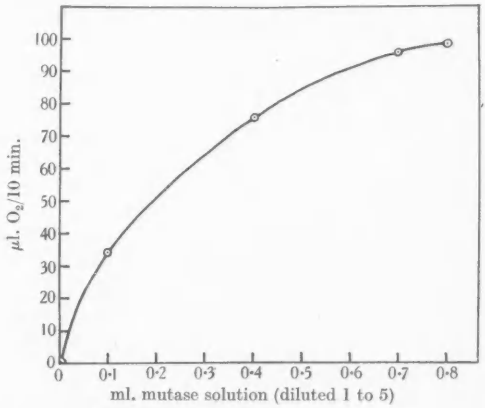


Fig. 3. The effect of varying the amount of mutase.

Each manometer contained 1·5 ml. factor, 1 ml. 0·15% coenzyme I, 0·3 ml. 0·5% methylene blue, 0·4 ml. *M*/10 pyrophosphate and 0·3 ml. *M*/10 hexosediphosphate. The centre pots were filled with KOH paper to absorb $CO_2$. Both factor and mutase were prepared from rabbit skeletal muscle.

If there are two catalysts involved in the oxidation of triosephosphate by methylene blue, we should expect that with a constant amount of dehydrogenase the velocity of oxidation would be proportional to the amount of coenzyme factor and vice versa. Figs. 2 and 3 show that such is indeed the case. It is obvious from the curves that the maximum activity of a given amount of dehydrogenase is independent of the amount of factor beyond a critical saturation concentration. In other words, these two catalysts although collaborating in series are independent of one another as far as their catalytic activity is concerned.

The factor solution used in the above experiments was tested for the presence of flavin as follows. The solution was centrifuged hard until the supernatant was water clear. The sediment was then shaken mechanically for 24 hr. with a mixture of 3 parts methyl alcohol to 1 part of water. The methyl alcohol extract of an amount of factor solution equivalent to 300 g. of fresh tissue contained no trace of flavin. As a control a solution of flavoprotein was tested for flavin under the same conditions and the extraction of flavin was complete. There can be no question therefore of the factor effect being due to traces of flavoprotein. But more important is the fact that flavoprotein has little activity as factor in the reconstructed system (cf. Table III). 1 mg. of crude heart factor has the same

Table III. *Flavoprotein as factor*

The system contained 1 ml. 0·15% coenzyme I, 0·3 ml. *M*/10 hexosediphosphate and 0·3 ml. 0·5% methylene blue. The other quantities were 1 ml. mutase, 1 crude heart factor and 1 ml. 2·5 mg. pure flavoprotein.

| | Methylene blue, reduction time in min. |
|---|---|
| Mutase + system | ∞ |
| Mutase + factor + system | 2 |
| Mutase + flavoprotein + system | 60 |
| Mutase + factor + flavoprotein + system | 2 |

activity as 70 mg. pure flavoprotein in the triosephosphate system and as 20 mg. in the malic system. Making the not unreasonable assumption that the purity of the crude factor solution is not >1%, weight for weight the factor is at least 2000–7000 times as active.

That the coenzyme factor is not identical with flavoprotein is quite definite. But it would be dogmatic to conclude from the inability of flavoprotein to replace the factor under the conditions of our experiments that flavoprotein plays no catalytic role at all. Before anything can be decided about the role of flavoprotein in the oxidative events of animal tissues, it would be well to know whether the flavoprotein which has been isolated from bottom yeast by Warburg & Christian [1932] has any counterpart in animal tissues. Free flavin is indeed found in abundance but there is not the slightest evidence yet presented that the Warburg-Christian flavoprotein as distinct from unspecifically "bound flavin" exists in animal tissues.

*Triose.*

The triose mutase catalyses the oxidation of glyceraldehyde to glyceric acid [cf. Green *et al.* 1937, 2]. The following experiment shows the effect of factor. The complete system containing 0·5 ml. mutase, 1 ml. factor, 1 ml. coenzyme I and 0·5 ml. 1% glyceraldehyde reduced 1 mg. methylene blue in 5 min. Without mutase, triose or coenzyme no reduction took place. Without factor complete reduction was reached in 50 min. Although the triose mutase in our preparations

is extremely active when tested by the usual anaerobic mutase oxidoreductions, poor results are obtained in aerobic experiments with the full system which consists of mutase coenzyme, factor and methylene blue. It is possible that traces of peroxide formed during the aerobic oxidation destroy the enzyme. In a later section the results are given of aerobic experiments in which methylene blue is replaced by the cytochromes.

*Lactic and malic.*

Both the triosephosphate and triose mutases catalyse the oxidation of their substrates by means of α-ketonic acids such as pyruvic and oxaloacetic acids. Since it is possible by means of the factor to study the triosephosphate mutase as a triosephosphate dehydrogenase, it should similarly be possible to study the oxidizing half of the mutase as a lactic or malic dehydrogenase. Table IV contains

Table IV. *Factor effect with lactic and malic enzymes associated with the muscle mutase*

The complete system contained 1·5 ml. mutase, 0·5 ml. factor, 1·0 ml. 0·15% coenzyme I, 0·2 ml. 0·5% methylene blue, 0·2 ml. 2 *M* HCN and 0·2 ml. *M* lactate or malate. Factor prepared from pig heart was used in the lactic experiments whereas factor prepared from rabbit skeletal muscle was used in the malic experiments.

Oxygen uptake in μl./10 min.

| | Lactic | Malic |
|---|---|---|
| Complete system | 276 | 78 |
| Without mutase | 5 | 0 |
| Without factor | 0 | 10 |
| Without substrate | 17 | 0 |
| Without coenzyme | 5 | 0 |

the results of typical experiments. When dealing with the lactic enzyme, heart factor was employed. But in the case of the malic enzyme, skeletal muscle factor was found to be more suitable. The choice of the factor solution has no relevance to the activity of the enzyme or to any possible differences between the factors prepared from the two sources. The factor solutions are always found to contain small amounts of dehydrogenases which defy the washing process. The amount in the final factor solution depends upon the concentration of the dehydrogenase in the original extract from the ground tissue. Thus since pig heart is relatively weak in the lactic dehydrogenase, the factor solution obtained from this source shows only a very slight lactic enzyme activity. Similarly, since the skeletal muscle of rabbit is relatively weak in the malic enzyme, the factor solution obtained from this source is suitable for tests with a malic enzyme. It is possible therefore by judicious selection of the source of factor to eliminate the blanks occasioned by the factor solution containing the particular dehydrogenase or mutase under study.

### IV. *The factor effect with coenzyme dehydrogenases*

When rabbit skeletal muscle is minced and mixed with water, the extract is found to be rich in the mutases referred to above, but practically free of the coenzyme factor. Mere washing of the tissue is insufficient to extract the factor. The essential requirement for extraction is that the tissue should be finely ground. The same is true of the coenzyme dehydrogenases, e.g. the lactic, malic and β-hydroxybutyric enzymes. It was natural therefore that for a long time the coenzyme dehydrogenases were credited with properties which were really

those of the coenzyme factor. The study of the mutases showed that the properties of, for example, the lactic enzyme prepared from ground muscle were different from those of the lactic enzyme associated with the mutase. The former apparently could catalyse the oxidation of reduced coenzyme by methylene blue whereas the latter could not except in presence of the factor. But experiment soon showed that this difference was not real and that the lactic enzyme regardless of the method of preparation lacked the power of catalysing the oxidation of reduced coenzyme.

Tables V and VI show that the lactic, malic, $\beta$-hydroxybutyric, alcohol and hexosemonophosphate dehydrogenases when separated from the coenzyme

Table V. *Factor effect with lactic and malic dehydrogenases*

The malic dehydrogenase was prepared from pig heart as follows. The washed heart mince was ground with sand and phosphate. The mixture was filtered through muslin. The filtrate was cooled to 0° and mixed with 3 vol. cold acetone. The precipitate was dried with acetone and ether and resuspended in phosphate buffer. The mixture was centrifuged and the supernatant filtered with suction through a pad of charcoal and kieselguhr. The factor was prepared by the usual method from rabbit skeletal muscle.

The lactic dehydrogenase was prepared from rabbit skeletal muscle by the method of Green & Brosteaux [1936]. The preparation was centrifuged hard and the supernatant heated at 55° for 30 min. The factor used was prepared from pig heart.

The following quantities were used in both the lactic and malic experiments: 1 ml. dehydrogenase, 1 ml. factor, 0·2 ml. 2 *M* HCN, 0·4 ml. *M*/10 pyrophosphate, 0·05 ml. 0·5% methylene blue and 0·2 ml. *M* substrate.

Oxygen uptake in $\mu$l./40 min.

| | Lactic | Malic |
|---|---|---|
| Complete system | 282 | 260 |
| Without dehydrogenase | 15 | 30 |
| Without factor | 40 | 30 |
| Without substrate | 0 | 0 |
| Without coenzyme | 0 | 0 |

Table VI. *The factor effect with the $\beta$-hydroxybutyric, alcohol and hexosemonophosphate dehydrogenases*

The $\beta$-hydroxybutyric dehydrogenase was prepared from pig heart by the usual method and heated at 55° for 15 min. The factor used was that of rabbit skeletal muscle. The complete system contained the following components: 1 ml. dehydrogenase, 1 ml. factor, 1 ml. 0·15% coenzyme I, 0·2 ml. *M*/2 hydrazine, 0·4 ml. *M*/10 pyrophosphate, 0·2 ml. *M dl*-$\beta$-hydroxybutyrate and 0·05 ml. 0·5% methylene blue.

The alcohol dehydrogenase was prepared from horse liver by the method of Dixon & Lutwak-Mann [1937]. The factor used was that of rabbit skeletal muscle. The complete system contained 1·5 ml. dehydrogenase, 1 ml. factor, 1 ml. 0·15% coenzyme I, 0·2 ml. 0·5% methylene blue and 0·4 ml. 98% ethyl alcohol.

The hexosemonophosphate dehydrogenase was prepared from the skeletal muscle of the rabbit by the same procedure as for the lactic enzyme. The factor used was that of rabbit skeletal muscle. The complete system contained 1 ml. dehydrogenase, 1·5 ml. factor, 0·2 ml. 0·5% methylene blue, 0·3 ml. *M*/3 hexosemonophosphate and 1 ml. 3% crude coenzyme II prepared from horse corpuscles.

Methylene blue, reduction time in min.

| | $\beta$-Hydroxybutyric | Alcohol | Hexosemonophosphate |
|---|---|---|---|
| Complete system | 5 | 0·5 | 10 |
| Without factor | ∞ | ∞ | ∞ |
| Without dehydrogenase | ∞ | ∞ | ∞ |
| Without substrate | ∞ | 25 | ∞ |
| Without coenzyme | ∞ | ∞ | ∞ |

factor are unable to catalyse the oxidation of their respective substrates by means of methylene blue. The lactic and hexosemonophosphate enzymes were prepared from ground and washed rabbit skeletal muscle by the methods described in previous communications, the malic and $\beta$-hydroxybutyric enzymes were prepared from pig heart muscle and the alcohol enzyme was obtained from horse liver by a method similar to that of Dixon & Lutwak-Mann [1937]. The detailed purification necessary for eliminating the factor is given in connexion with the appropriate table. Oxygen uptakes were measured whenever the enzyme studied was sufficiently active. With enzymes of comparatively weak activity, the methylene blue technique was employed. The hexosemonophosphate dehydrogenase of skeletal muscle requires the presence of coenzyme II. The strikingly positive factor effect shows clearly that the factor catalyses the oxidation of reduced coenzyme II, and that even in the case of coenzyme II dehydrogenases flavoprotein is not an essential component.

It will be observed that pyrophosphate is added in many cases in addition to the enzymes, coenzyme and methylene blue. Green *et al.* [1937, 1] showed that pyrophosphate exerted a stabilizing effect on the $\beta$-hydroxybutyric dehydrogenase in the sense that the initial velocity was maintained for a longer period in presence of the reagent than in its absence. We have found that this peculiar effect of pyrophosphate is shown in the cases of practically all coenzyme-dehydrogenase systems. Pyrophosphate is, of course, not necessary for demonstrating the factor effect but it is of value in prolonging the period during which the enzymes still retain catalytic activity.

### V. *Distribution of the factor*

We have not as yet carried out any systematic investigations of the distribution of the coenzyme factor in animal tissues. Some experiments with the tissues of the rabbit show that the enzyme is present in brain, heart, skeletal muscle, kidney and liver. The first three tissues were rich in the coenzyme factor, whereas liver and kidney contained smaller amounts.

The factor is prepared from the various tissues by the method outlined in section II. The presence or absence of the factor is determined by the catalytic effect on the reaction between reduced coenzyme and methylene blue (cf. section I).

### VI. *The reaction of coenzyme systems with cytochromes* a *and* b

The triosephosphate mutase system in presence of the factor from pig heart takes up oxygen in absence of any added carrier. On addition of methylene blue or pyocyanine this uptake is doubled or trebled. These facts are consistent with the view that the factor solution contains a natural carrier albeit in small concentration. Green *et al.* [1937, 1] have already observed this direct reaction with molecular oxygen in absence of added carrier in the cases of the lactic, malic and $\beta$-hydroxybutyric dehydrogenases. They used the preparation from pig heart as the source of these enzymes. The direct reaction with oxygen was inhibited completely by $M/100$ HCN. Furthermore the enzyme preparation after precipitation with acetone failed to react with oxygen unless some carrier was added.

Spectroscopic examination of the factor solution prepared from pig heart discloses the presence of cytochromes *a* and *b*. A mixture of the triosephosphate mutase, heart factor and coenzyme I shows no bands of the reduced cytochromes. On addition of triosephosphate the bands of reduced cytochromes *a* and *b* appear instantaneously (cf. Table VII). This experiment can be repeated with lactic and

Table VII. *Reduction of cytochromes* a *and* b *by the triosephosphate mutase-factor system*

The experiment was carried out in evacuated Thunberg tubes at room temperature. The reduction was observed with a microscope-spectroscope using a Pointolite as source of illumination.

| | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Mutase | 0·1 | — | 0·1 | 0·1 |
| Factor | 2·0 | 2·0 | 2·0 | 2·0 |
| Coenzyme | 0·2 | 0·2 | 0·2 | — |
| *M*/10 hexosediphosphate | 0·3 | 0·3 | — | 0·3 |
| Water | — | 0·1 | 0·3 | 0·2 |
| Reduction time of cytochromes *a* and *b* | Instantaneous | Partial reduction in 10 sec. | ∞ | ∞ |

malic acids and glyceraldehyde as substrates with the same results. The mutase solution provides the enzyme for reducing the coenzyme while the factor catalyses the reaction between reduced coenzyme and the cytochromes.

The factor solution prepared as in section II is relatively weak in the cytochromes. Prof. D. Keilin and Dr Hartree have kindly informed us of a simple method of obtaining a factor solution extremely rich in cytochromes *a* and *b*. The thoroughly washed and minced heart muscle is ground in a mechanical mortar for *ca.* 2 hr. until a completely homogeneous cream is formed. Instead of straining the mixture through muslin as in the usual preparation, the cream is centrifuged and the top fluid part is poured off and brought to *p*H 4·6. The sediment after precipitation is resuspended in buffer and used directly. Since this method does not involve prolonged washing of the insoluble particles the final preparation has high lactic, malic and $\beta$-hydroxybutyric activities. Fortunately, triosephosphate and triose activities are practically nil. It should be pointed out that cytochromes *a* and *b* as well as the cytochrome oxidase and the factor are all associated with insoluble particles and invariably accompany one another.

Figs. 4 and 5 show the complete cytochrome reconstructions for the triosephosphate and triose systems respectively. The velocities of these reactions are much higher than those in presence of methylene blue, flavin or pyocyanine as carriers. Furthermore, the reactions are more linear with time and fail to show the rapid falling off in activity which characterizes experiments with artificial carriers. From a rough estimation of the amount of cytochrome present in the factor preparation, it would appear that each molecule of cytochrome is reduced and oxidized at least a thousand times per minute.

Cytochrome *c* appears to take no part in the reaction between reduced coenzyme and oxygen. Addition of comparatively huge amounts to the reconstructed system has no effect on the velocity. This is in agreement with the fact that the reaction between reduced coenzyme and cytochrome *c* is extremely slow compared with the reaction with cytochromes *a* and *b*. When the concentration employed of cytochromes *a* and *b* is considerable as in the reconstruction experiments, the addition of other carriers such as methylene blue has no influence on the velocity.

Table VIII shows that the other coenzyme systems can react vigorously with oxygen via the cytochrome path. Since the factor solution already contains the malic and $\beta$-hydroxybutyric enzymes, only substrate, coenzyme and ketone fixative need be added. In the case of the experiment on the lactic enzyme mutase solution is also added in order to ensure an adequate concentration of the lactic enzyme.

The diagnostic test of the working of the cytochrome-cytochrome oxidase system is the sensitivity to low concentrations of cyanide. It is obviously impossible to use cyanide as an inhibitor in presence of glyceraldehyde or triosephosphate because of irreversible cyanohydrin formation and consequent

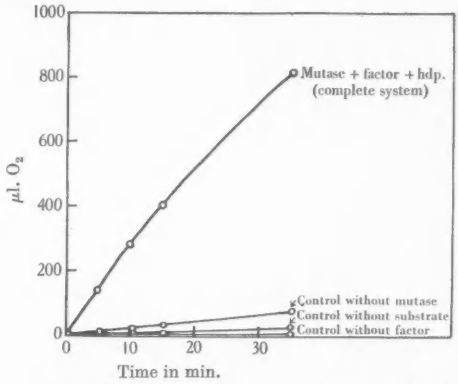


Fig. 4. The direct reaction between the triosephosphate system and molecular oxygen in absence of added carrier.

The following quantities were used in the complete system: 1 ml. mutase, 1·5 ml. factor, 1 ml. 0·15% coenzyme I, 0·4 ml. *M*/10 pyrophosphate and 0·3 ml. *M*/10 hexosediphosphate. The centre pot of each manometer was filled with KOH paper to absorb $CO_2$. The mutase was prepared from rabbit skeletal muscle and the factor from pig heart by the special method of Keilin and Hartree.

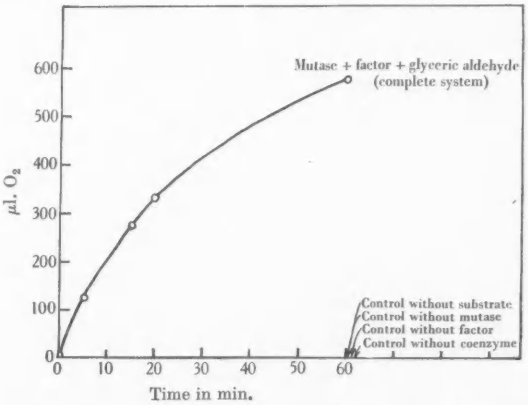


Fig. 5. The direct reaction between the triose system and molecular oxygen in absence of added carrier.

Details as for Fig. 4 except that 0·2 ml. 5% *dl*-glyceraldehyde was used as substrate.

removal of free cyanide. *M*/100 cyanide completely inhibits the oxidation of lactic acid whereas *M*/500 inhibits to the extent of about 65%. The cytochrome oxidase, however, should be completely poisoned at *M*/1000 cyanide concen-

Table VIII. *The reaction of coenzyme systems with molecular oxygen via cytochromes* a *and* b

The complete system in all cases contained 1·5 ml. factor, 1 ml. 0·15% coenzyme I, 0·4 ml. *M*/10 pyrophosphate, 0·2 ml. *M*/2 hydrazine and 0·2 ml. *M* substrate. In the case of the lactic experiment 0·5 ml. of muscle mutase was also added to provide additional enzyme.

| | $\mu$l. $O_2$ in 40 min. |
|---|---|
| Complete lactic system | 542 |
| Without coenzyme | 0 |
| Without substrate | 10 |
| With *M*/100 HCN | 40 |
| With *M*/500 HCN | 181 |
| Complete malic system | 290 |
| Without coenzyme | 0 |
| With *M*/100 HCN | 25 |
| Complete $\beta$-hydroxybutyric system | 340 |
| Without coenzyme | 0 |
| With *M*/100 HCN | 27 |

tration. For some time we considered this discrepancy to mean that the cytochrome oxidase was not involved in the direct reaction with oxygen. But further consideration has disclosed a valid reason for this discrepancy. Lactic acid on oxidation yields the corresponding ketonic acid which can combine with cyanide. The oxidation of lactic acid by the coenzyme is a process which proceeds independently of the oxidation of reduced coenzyme by the cytochromes. That is to say, in presence of a given quantity of coenzyme a certain amount of pyruvic acid must be formed depending upon the equilibrium. Using *M*/1000 final concentration of inhibitor, the amount of cyanide available in the manometer is of the order of magnitude of the pyruvic acid formed. The effective concentration of cyanide is therefore much lower and the inhibition is correspondingly less. Furthermore, if the reduced coenzyme can be oxidized even to the extent of 2–3 $\mu$l. of oxygen equivalence by traces of an oxidizing agent in the preparation, an amount of pyruvic acid would be formed sufficient to reduce the effective cyanide concentration to a level at which inhibition would only be partial. Unfortunately all the coenzyme systems available for study involve either the oxidation of an aldehyde or the production of a ketonic acid. In both cases the difficulty of cyanide fixation cannot be avoided.

When the cytochrome-containing factor solution is precipitated with acetone at 0°, dried and resuspended in buffer, no direct reaction with oxygen takes place except in presence of added carriers. It is obvious that acetone treatment destroys the components necessary for the cyanide-sensitive reaction. Spectroscopic examination discloses considerable destruction of components *a* and *b*. Furthermore, all cytochrome oxidase activity is lost. This correspondence between the disappearance of the direct reaction and the destruction of the cytochrome oxidase system may be fortuitous. But there are other striking illustrations of this parallelism. For example, factor preparations such as that of rabbit skeletal muscle which are relatively deficient in the cytochromes show only a weak reaction with oxygen in absence of added carriers.

We may summarize the various lines of evidence in favour of the view that cytochromes *a* and *b* are the carriers in our preparation which account for the direct reaction with molecular oxygen.

(1) Cytochromes *a* and *b* are rapidly reduced by all coenzyme systems in presence of the factor.

(2) The direct reaction is poisoned completely by $M/100$ cyanide and to the extent of 65% by $M/500$ cyanide. The cytochrome oxidase is the only cyanide-sensitive enzyme known to be present in the reconstructed system.

(3) Procedures which destroy the cytochromes or the oxidase eliminate the direct reaction with oxygen.

(4) The velocity of the direct reaction is proportional to the amount of cytochromes *a* and *b* in the factor solution.

### VII. *General considerations*

Strictly speaking, the coenzyme factor is a dehydrogenase which catalyses the oxidation of its substrate, i.e. reduced coenzyme by means of various hydrogen acceptors. But to refer to the coenzyme factor as "coenzyme dehydrogenase" would introduce confusion with "coenzyme dehydrogenase systems". It is preferable that the term dehydrogenase should be applied only to enzymes which are concerned in catalysing the oxidation of metabolites. For the moment at any rate, in lieu of a more suitable name, the enzyme which catalyses the oxidation of reduced coenzymes I and II will be referred to as the coenzyme factor.

There are various lines of evidence that the coenzyme factor is definitely an enzyme. It is destroyed by 15 min. exposure to 55° and by a few minutes exposure to strong acid or alkali. It is non-dialysable and is associated with insoluble particles of a protein nature. Incubation of the factor solution with trypsin at 38° gradually destroys all enzymic activity. As yet there is nothing known of the mechanism whereby the coenzyme factor produces its catalytic effect. Purified factor solutions from rabbit skeletal muscle show little colour apart from that due to traces of the cytochromes. There is therefore no basis as yet for postulating that the factor like flavoprotein undergoes reversible oxidation and reduction.

Flavoprotein has $<1/1000$ the activity of coenzyme factor in any of the systems which we have studied in animal tissues. But flavoprotein definitely can act in the capacity of a carrier in the same way as flavin or adrenochrome. The catalytic efficiency of flavoprotein is about of the same order as that of adrenochrome (turnover number 5) but much less than that of cytochromes *a* and *b*, the turnover number of which lies in the thousands [cf. Green & Richter, 1937; Haas, 1934; Warburg, 1934; Warburg & Christian, 1933].

That the cytochromes are the normal physiological carriers for the coenzyme systems is borne out by the following facts. The oxidation of the substrates of coenzyme systems in tissue slices is inhibited completely by dilute cyanide [cf. Green & Brosteaux, 1936; van Heyningen, 1935]. The same cyanide-sensitivity is true of yeast [Ogston & Green, 1935]. Furthermore, Haas [1934] has calculated the percentage of the total respiration of yeast which takes the cytochrome path, and from his data it would appear that within the limits of experimental error the entire respiration involves the reversible reduction and oxidation of the cytochromes.

Prof. D. Keilin has informed us that he has invariably observed in yeast and in animal tissues rapid reduction of the cytochromes by the substrates of coenzyme systems. It was in fact Prof. Keilin's insistence on the participation of the cytochromes in coenzyme phenomena that induced us to correlate the cyanide-sensitive reaction with the cytochrome system.

The cytochrome class of dehydrogenases has been defined by Green & Brosteaux [1936] as one which catalyses the direct oxidation of the substrate by the cytochromes (*a*, *b*, or *c*). The coenzyme dehydrogenases are sharply

separated from this class in that the reaction between substrate and cytochrome is indirect—the coenzyme being the intermediary. Furthermore, the coenzyme dehydrogenases are not concerned with the reduction of cytochrome—that being the function of the coenzyme factor. The distinction drawn between the cytochrome and coenzyme systems is still valid. In the former class cytochrome is the oxidant for the substrates; in the latter the coenzyme specifically fulfils that function.


## Summary

1. The isolation and properties of the coenzyme factor are described. This enzyme catalyses the oxidation of reduced coenzymes I or II by hydrogen acceptors such as flavin, cytochrome, adrenochrome etc.

2. Coenzyme dehydrogenases from animal tissues have been shown to take part only in the reaction between the substrate and the coenzyme. In absence of coenzyme factor no reaction with oxygen via carriers is possible.

3. Flavoprotein has $< 1/1000$ the activity of the factor in coenzyme I systems.

4. A system in which the substrates of coenzyme dehydrogenases are oxidized by way of cytochromes *a* and *b* has been reconstructed from natural components.


One of us (D. E. G.) is grateful to the Ella Sachs Plotz Foundation for a research grant. We should like to take this opportunity of thanking Prof. Sir F. G. Hopkins for the interest he has shown in the progress of this work. Dr C. Lutwak-Mann has kindly carried out for us experiments on the alcohol dehydrogenase designed to show the factor effect.

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